



THE  
INDIAN JOURNAL  
OF  
MEDICAL RESEARCH

INDIAN MEDICAL COLLEGE,  
L. R. D. Y.,  
J. F. No...7315..  
...9...10...6..



PUBLISHED FOR  
THE INDIAN RESEARCH FUND ASSOCIATION  
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# JOURNAL OF THE MALARIA INSTITUTE OF INDIA

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## THE IMMUNOLOGICAL SKIN TESTS IN LEPROSY

### Part I

#### THE ISOLATION OF A PROTEIN ANTIGEN OF *MYCOBACTERIUM* *LEPRÆ*

BY

DHARMENDRA, M B, B S, D B

(From the Leprosy Inquiry, Indian Research Fund Association, School  
of Tropical Medicine, Calcutta)

[Received for publication, September 25, 1941]

#### INTRODUCTION

*The lepromin test*—Mitsuda reported in 1916 that intracutaneous injections of an emulsion of boiled leprous tissue rich in bacilli usually produced no reaction in 'nodular'\* leprosy but a marked local reaction in 'neuro-macular'\* leprosy. The local reaction took the form of a nodule in the skin and usually appeared in the second or third week. At the International Leprosy Conference, Strasbourg, Mitsuda (1924) reported positive results in 'neuro-macular' cases, in healthy contacts and in non-contacts, and negative results in 'nodular' cases. Many workers have since confirmed Mitsuda's findings. The test has been known as lepromin test, or the Mitsuda reaction after the originator.

*The nature of the reaction*—The test has been investigated widely and during recent years the literature on the subject has become very extensive. The mechanism of the reaction however, is not clear. Mitsuda interpreted a positive reaction as indicating the resistance of the tissues (in healthy individuals and 'neural' cases of leprosy) to the injected bacilli, and a negative reaction as showing lack of resistance of the tissues (in 'lepromatous' cases of leprosy). He did not consider a positive reaction as having been caused by exposure to infection. Most workers have adopted the main position of Mitsuda regarding the non-specific nature of the reaction. Some workers, however, maintain that a positive reaction is one of specific allergy caused by exposure to infection.

---

\* The types are now called 'lepromatous' and 'neural', respectively



It is difficult to reconcile this view with the fact that positive results have been reported in people who have never been exposed to leprous infection. In view of this fact and of the fact that a positive test is otherwise strongly suggestive of an allergic phenomenon, a few workers have considered a positive test as allergic but not necessarily specific.

Another anomalous feature of the test is that people suffering from the more serious type of leprosy (the 'lepromatous' type) usually give negative results.

*The lateness of the reaction* —In addition to the lack of specificity, the lateness of the reaction is another feature which distinguishes it from the allergic skin reactions, the classical Mitsuda reaction beginning not earlier than the end of the first week, and not reaching its maximum before the third or fourth week. Early reactions of 'tuberculin' type (24 to 48 hours) are however, sometimes seen. The early reaction is discussed later.

*The cause of the delayed reaction* —The extraordinary delay in the reaction has aroused surprisingly little comment and few workers have attempted any explanation. It appeared to the author that the delay in reaction to lepromin is caused by the nature of the material injected to elicit the reaction. Whereas in the other allergic skin tests a soluble antigen, which is free to act at once is injected, in the lepromin test whole bacilli (together with the nodular tissue) are injected, the antigen being liberated slowly in minute amounts over a prolonged period.

*The need for a soluble antigen* —A soluble and refined antigen, if available, could therefore be expected to throw much light on the mechanism of the test. The extreme crudeness of the antigen may also perhaps be responsible for the production of positive results in the individuals not exposed to infection. If so with a refined antigen, the test may possibly be made specific.

*Previous attempts to obtain a soluble antigen* —Some workers have prepared antigenic fractions of the leprous nodule, not of the bacilli themselves. Their work has provided no new information about the mechanism of the test. This work is discussed later.

*The present attempts* —It was considered that attempts should be made to get an antigenic fraction from the bacilli themselves, and not from the leprous nodule. The work reported in this publication was undertaken with a view to obtaining a soluble antigen of *Mycobacterium lepræ* similar to the T P T (tuberculin-protein trichloroacetic acid precipitated) of *Mycobacterium tuberculosis* although in the absence of a culture of *Myco lepræ* the methods employed would naturally differ.

The results reported herein would show that the attempt has succeeded. A soluble antigen, protein in nature, has been isolated and the results of the tests performed with the antigen throw much light on the mechanism of the reaction. The isolated antigen, it is believed, will greatly facilitate immunological studies in leprosy.

## PRESENT WORK

*The separation of the bacilli*—In the absence of a culture of *Mycobacterium leprae* the source of such an antigen has naturally to be the lepromatous tissue. The first step was, therefore, to obtain leprosy bacilli free from tissue. If this could be done, the active principle could then be separated from the bacilli. Attempts to separate the bacilli from the tissue by digestion of the leprosy nodules with *papain* and by fractional centrifugalization of suspensions of the nodules in saline did not succeed. Centrifugalization succeeded up to a point, a suspension consisting of single bacilli and only traces of very fine tissue being obtained. The bacilli from this suspension could not, however, be thrown down even by prolonged centrifugalization at 6 000 r p m (the highest speed available), since even at that speed most of the single bacilli remain in suspension. The addition of various chemical substances to lower the surface tension of the fluid previous to centrifugalization also failed to produce the desired result. It was, however, noted that when the lepromin (in saline) was shaken with chloroform and the two fluids allowed to separate on standing, almost all the bacilli were found in the chloroform layer. Ether and carbon tetrachloride used similarly were not so satisfactory. This special affinity of chloroform for the bacilli has been utilized to obtain bacilli from the lepromatous tissue.

The pieces of lepromatous material, usually nodules cut from ears, are autoclaved and are ground up with chloroform in a glass pestle and mortar. The chloroform is pipetted off. The grinding in chloroform is repeated till a smear from the remaining tissue is almost free from bacilli. (About 50 c c of chloroform are necessary to extract almost all the bacilli from 2 g of lepromatous tissue.) All the lots of chloroform used in grinding are pooled, and the remaining tissue is discarded. A smear from the pooled lot of chloroform shows bacilli in very large numbers and the absence of any tissue.

The chloroform is then completely evaporated over a water bath, the residual substance consists of lipoids and bacilli. The residue is then suspended in ether and the ethereal suspension is centrifugalized at a low temperature at 3,000 r p m. (In this hot climate this is done in a refrigerator.) The lipoids remain in the supernatant ether and the bacilli are deposited at the bottom. To remove the lipoids more completely the bacillary deposit is again suspended in ether, the suspension centrifugalized and the deposited bacilli separated and dried. Smears made from the dried powder show only bacilli and no tissue. (The bacilli constitute about 0.4 per cent of the weight of the nodules.) The supernatant ether on evaporation leaves a deposit of the lipoids.

*Antigenic activity of the three constituents of the nodule*—The antigenic activity of the three different constituents of the leprosy nodule (the bacilli, the tissue lipoids and the residual tissue after chloroform treatment) was then tested in cases of leprosy of the 'neural' type for it is in this type of the disease that lepromin gives positive results. Suspensions in carbol-saline were prepared from all the three constituents, the strength of all the suspensions being 1 mg of the substance to 1 c c of saline. Equal amounts of these three suspensions were injected into the skin of the patients and readings of the results were made at 24 hours, 48 hours and 1 week and thereafter weekly for several weeks.

From the results obtained it was concluded that the bacilli were the only definitely antigenic constituent of the leprosy nodule producing both the early (tuberculin type) and the late (nodular) reactions. In highly reactive cases the lipoids, and to a much less extent the tissues, gave rise to a slight reaction most probably caused by the traces of the bacillary antigen remaining in the other two constituents of the nodule.

*The isolation of the different chemical fractions of the bacillus*—The bacilli having thus been obtained free from tissue and been found to be the only definitely antigenic constituent of the leprous nodule, the next step was to isolate the different chemical fractions of the bacillus itself in order to find out which of these were antigenic

As a preliminary to isolating the different fractions, the bacilli were thoroughly broken down by mechanical means (grinding). The grinding was done in an agate mortar with the addition of very small amounts of saline from time to time. This is a very long and laborious process. It takes several hours to destroy the bacillary form and acid fastness of even minute amounts of the material. The grinding has been done inside a glass-covered box to avoid contamination. It has been found that the addition of a few spots of chloroform from time to time aids grinding and may help in maintaining sterility (The chloroform quickly evaporates)

The ground bacilli were then extracted with 0.5 per cent carbolic saline, the ground up material being shaken up with the saline and the suspension allowed to stand overnight. The supernatant fluid was pipetted off and constituted the saline soluble portion of the bacilli, the bulk of the residue being the saline insoluble portion.

The saline extract was centrifugalized to throw down any suspended matter. The supernatant fluid gave positive Biuret and Molisch's reactions. It was, therefore, concluded that the extract contained protein, and polysaccharides either free or linked to protein.

The protein was precipitated from the extract by precipitation with trichloroacetic acid. An equal volume of 20 per cent trichloroacetic acid was added to the saline extract, and the mixture allowed to stand overnight. The next morning the precipitated protein was separated from the supernatant fluid. The precipitate was then washed in ether to remove traces of the trichloroacetic acid.

Polysaccharide was isolated from the supernatant fluid remaining after protein precipitation. Nine volumes of 95 per cent alcohol and a few crystals of sodium acetate were added to this fluid and the mixture allowed to stand overnight in the refrigerator. The precipitate of the polysaccharide was separated by removing most of the fluid by decantation and then by centrifugalizing the bottom portion of the fluid.

The residue left after saline extraction was washed several times with saline to remove as much as possible of the soluble portion retained in it. The different lipid fractions were then extracted from the residue by methods similar to those used by Anderson (1927) for the separation of lipid fractions from tubercle bacilli. The methods consist essentially in extracting the material with ether, treating the ethereal extract with acetone, and extracting the ether insoluble portion with chloroform. By such methods three lipid fractions were isolated: ether and acetone soluble (glycerides), ether soluble but acetone insoluble (phosphatides), and ether insoluble, chloroform soluble (waxes). Some residue was left after the chloroform extraction, the chemical nature of this residue is so far not known.

The following fractions were thus isolated from the leprosy bacillus —

(a) Protein, (b) polysaccharide, (c) glycerides, (d) phosphatides, (e) waxes and (f) final residue

These fractions were tested with the results described later

### *The antigenic activity of the bacilli and their different fractions*

(a) *Tests with ground and unground bacilli*—The results of comparative tests done on patients of the 'neural' type of leprosy with ordinary lepromin, whole bacilli and ground bacilli showed that —

- (i) Ground bacilli gave marked early and little late reactions
- (ii) Unground bacilli gave fairly-marked early and late reactions
- (iii) Ordinary lepromin gave less-marked early reactions and well-marked late reactions

Thus, the grinding of the bacilli was found to enhance the early reaction and reduce the late reaction. It appeared that by the breaking down of the bacilli a

portion of antigen is liberated in a soluble form, less of it being available to be liberated later

(b) *Tests with the saline extract of ground bacilli and the residue* —Comparative tests done on patients of leprosy of both the types (the 'neural' and the 'lepromatous') with the saline extract of ground bacilli and the residue gave the following results —

In the lepromatous cases no reaction, early or late, was produced by either of the preparations

In the neural cases the soluble portion usually produced early reactions only, whereas the insoluble portion produced both the early and the late reactions, although the late reactions were much weaker than those produced by ordinary lepromin or by ground bacilli (The results of the tests with the different fractions of the bacillus reported later have shown that the activity of the residue was mostly if not entirely, caused by retention of some of the soluble portion in the residue. The antigenic substance appears to be very firmly bound up with the other constituents of the bacterial cell since even after prolonged grinding it is impossible to extract all the antigenic substance from the insoluble residue)

The saline extract having thus been found definitely antigenic, some of its chemical, physical and biological properties were studied with the following results —

- (i) As already stated the solution contains protein and polysaccharides
- (ii) The soluble antigen is thermostable, as the solution is antigenically active after being sterilized in the autoclave at 120°C for half an hour
- (iii) It can pass through a Sietz filter although the degree of the reaction with the filtered material is much reduced
- (iv) The solution gives positive precipitin tests with some of the sera from cases of the 'lepromatous' type
- (v) With keeping, the saline extract loses some of its potency

(c) *Tests with the different bacillary fractions* —Protein, polysaccharide, the different lipid fractions and the final residue were tested in cases of leprosy of both the types (the 'neural' and the 'lepromatous') and the following results were obtained\* —

In the lepromatous cases no reaction early or late, was produced by any of the preparations

In the neural cases, the protein produced marked early but no late reactions. None of the lipid fractions produced any significant reaction, early or late. The

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\* The solution of protein and of polysaccharide and the suspensions of the other fractions were made of the same strength 0.1 mg. of the substance in 1 c.c. of carbol saline. In making the suspension the substance was put in a mortar, a few drops of N/10 NaOH were added, the material was ground with a pestle, and the required amount of carbol saline added. 0.2 c.c. of the solution or suspension (containing 0.02 mg. of the substance) were used for the test

polysaccharide and the residue left after extraction of the lipoids produced slight early reactions in some cases

Thus, the protein was demonstrated to be antigenic, and possibly the only antigenic fraction of the bacillus, producing only an early reaction of the 'tuberculin' type. The weak antigenic activity of the polysaccharide fraction is most probably caused by traces of the protein in it. The weak antigenic activity of the final residue can be accounted for in either or both of the following ways: (i) it may contain traces of the soluble antigen, (ii) it may contain another weak antigen.

(d) *Tests with the different fractions of the bacillary protein*—By extracting different lots of ground bacilli with weak acid, weak alkali and 80 per cent alcohol, three different proteins—acid-soluble protein, nucleo-protein and alcohol-soluble protein—have been isolated. All these protein fractions produce early reactions of the 'tuberculin' type in the neural cases of leprosy. The methods of their isolation and the details of their antigenic activity will be considered in a future publication.

#### SUMMARY.

1 The main features of the lepromin test (the Mitsuda reaction) are outlined. It is considered that the delay in the appearance of the classical nodular reaction, and possibly the non-specific nature of the reaction may be caused by the nature of the material injected. The need for a refined antigen is stressed.

2 A method for obtaining from excised nodules, leprosy bacilli free from tissue, is described. Bacilli have been found to be the active constituents of lepromin.

3 The bacilli were ground for several hours in an agate mortar and were fractionated into a saline-soluble portion and an insoluble residue. From the soluble portion, protein and polysaccharide have been obtained, from the insoluble residue, various lipid fractions have been separated.

4 Tests with the different fractions of the bacilli have shown that of all the fractions isolated, only the protein is definitely antigenic and that it produces only an early reaction.

5 By extracting the ground bacilli with different solvents, three protein fractions—acid-soluble protein, nucleo-protein and alcohol-soluble protein—have been isolated. All the three fractions give rise to early reactions in the neural cases of leprosy.

#### ACKNOWLEDGMENTS

My thanks are due to Dr J. Lowe, Officer-in-Charge of the Leprosy Department, School of Tropical Medicine, Calcutta, for his interest and useful suggestions, to Dr N. Mukerji of the same department for assistance in doing and reading the tests, to Mr K. S. Malik, Chemist of the Cholera Bacteriological Inquiry, Indian Research Fund Association, for valuable advice on the isolation

and fractionation of the bacilli, and to Mr R Bose of the Leprosy Inquiry, Indian Research Fund Association, for help in isolating and fractionating the bacilli

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## THE IMMUNOLOGICAL SKIN TESTS IN LEPROSY

### Part II

#### THE ISOLATED PROTEIN ANTIGEN IN RELATION TO THE CLASSICAL MITSUDA REACTION AND THE EARLY REACTION TO LEPROMIN

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[Received for publication, September 25, 1941 ]

#### *Three types of reaction*

In the present series of articles and in the literature of leprosy, intradermal injections of various preparations from leprous material have been reported as causing three different types of reaction

(a) *The classical Mitsuda reaction* —The main features of the classical Mitsuda reaction have been briefly outlined in Part I of this series (Dharmendra, 1942) Clinically, it is characterized by the formation of a definite nodule, sometimes accompanied by ulceration at the site of the intradermal injection of lepromin. This reaction is best seen three or four weeks after the injection

(b) *The early reaction preceding the classical reaction* —An early reaction is sometimes seen one or two days after the injection of lepromin and preceding the classical late reaction. Clinically this early reaction is quite different from the



classical Mitsuda reaction, for it is of the 'tuberculin' type and consists of an area of erythema accompanied by some oedema and thickening of the skin

Most workers have ignored these reactions as being of no significance but Fernandez (1940) made a special study of the early reaction. He found that it was always present in cases giving a marked late reaction and concluded that results of the early reaction are of the same significance as the late reaction. The findings of Fernandez regarding the significance of the early reaction have been confirmed by Lowe and Dharmendra (1941)

(c) *Early reaction followed by little or no late reaction*—Fernandez (*loc cit*) reported that the injection of a filtrate from lepromin induced an early reaction followed by no late reaction. This finding of Fernandez has also been confirmed by Lowe and Dharmendra (*loc cit*), although filtration was found to reduce the number and degree of definite reactions. The Joint Committee on Leprosy Skin Tests (1940) reported some early reactions followed by no late reaction to a protein isolated from leprosy spleen by Henderson (1940). Kitano and Inoue (1941) have reported early reactions unaccompanied by any late reactions to filtrate of lepromin treated by ultra-supersonic waves to break the bacilli contained in it. Early reactions, unaccompanied by any late reaction to a protein isolated from the leprosy bacillus, have been reported by one of us (D) in the preceding article (*loc cit*)

Thus, intradermal injections of the preparations derived from human material are capable of causing reactions of three different clinical types, the classical Mitsuda reaction, the early reaction preceding the classical reaction and the early erythematous reaction followed by no late reaction

### *The different reactions are caused by the same antigen*

In Part I it has been shown (a) that the lepra bacilli are the only antigenic element contained in lepromin and (b) that the protein is the only definitely antigenic fraction of the bacillus. As shown later all the three different types of reaction can be explained on the basis of this one antigen. It is therefore believed that all the three different types of reaction described are actually produced by the bacillary protein

### *Is there more than one antigen?*

Fernandez (*loc cit*) made a comparative study of the early and late reaction to lepromin and to filtrate from that preparation. He found that the ordinary lepromin produced late reactions in all the cases in which it had produced early reactions. The filtrate, however, usually produced the early reactions only. From these results Fernandez concluded that 'early and late reactions are probably brought about by different substances or toxins of the Hansen bacillus'

The reported work in the preceding article does not confirm the existence of the two antigens, one responsible for the early reaction and the other for the late. This work shows two things. Firstly, that to explain the early and late reactions

it is not necessary to postulate the presence of two antigens, secondly, that none of the chemical fractions obtained from the leprosy bacillus produces a late reaction

The findings of Fernandez can very well be explained on the basis of only one antigen, the early reaction being caused by the free antigen in the injected material and the late reaction by the same antigen which is slowly liberated from breaking down of the bacilli contained in that material. This view is suggested by the finding that the grinding of the bacilli enhances the early and reduces the late reaction, the preliminary breaking down of the bacilli being accompanied by the liberation of a large amount of the antigen leaving less antigen to be liberated later. The work on the fractionation of the bacillus supports this view.

Our first attempts at fractionation of the bacillus into soluble and insoluble portions gave results which were not clear-cut, for while the saline extract of the bacilli gave rise to early reactions, the bacterial residue left after thorough grinding and extraction with saline was still antigenically active, producing both the early and late reactions. (The degree of late reaction was, however, much reduced.) This observation could have been interpreted as showing the existence of an antigen other than the one removed by saline extraction. More thorough fractionation of the bacillary powder has, however, shown that most, if not all, of the activity of the insoluble residue had been caused by incomplete fractionation. A thorough fractionation of the bacillary powder has shown that none of the isolated fractions nor the final bacterial residue gives rise to a late reaction.

Another worker has brought up the question of the plurality of antigens in quite a different sense. De Souza Lima (1938) suggested that the Mitsuda antigen is a three-part complex, one part coming from the tissue cells and being non-specific, a second part being common to the acid-fast bacilli in general, and a third part being specific for the leprosy bacillus. We have shown that the tissue cells completely freed from the bacilli are not antigenic. The statement of De Souza Lima regarding the part played by the tissue cells in bringing about the Mitsuda reaction has, therefore, not been confirmed. It has been shown that the whole Mitsuda reaction depends on the bacilli. The present work has shown that the protein antigen can be divided into at least three fractions, it is not impossible that one or more of these fractions is 'species specific' and one 'type specific'. This conception is, moreover, in keeping with knowledge of the antigenic make up of other bacteria.

Thus, our work disproves the presence of two antigens in the sense indicated by Fernandez (one antigen for the early reaction, and the other for the late) but shows that there is more than one antigen of protein nature.

#### *The test with the isolated antigen and a comparison with the Mitsuda test*

The early reactions to the soluble antigen have been observed in 125 cases of leprosy, some 'neural' and some 'lepromatous'.

In this work the antigen was used in doses of 0.02 mg., and positive results were obtained in most of the cases in which they could be expected. The not

infrequent occurrence of focal reactions however and the persistence of the early reaction for a week or more, have suggested that the dose used was too large and that in cases of leprosy (but perhaps not in contacts), a considerably smaller dose could be used. In reading the results of this test a definite area of erythema of 10 mm or more in diameter accompanied by infiltration and cedema has been recorded as a positive result. The average diameter of the erythema in positive cases was over 15 mm, the maximum being over 40 mm. The reading is best made at 24 hours.

Positive results were seen in cases of the 'neural' type and negative results in cases of the 'lepromatous' type. The significance of the test with this antigen, therefore, appears to be the same as that of the classical Mitsuda reaction.

A comparative study of the early reaction to the soluble antigen and of the late reaction to ordinary lepromin (the Mitsuda reaction) in the same patients has shown that the isolated antigen is at least as sensitive as, if not more sensitive than, ordinary lepromin.

The test with the isolated antigen has, moreover, great advantages over the classical Mitsuda reaction —

- (1) A pure antigen is used, which is of a known chemical nature and which can be accurately standardized by weight. This antigen is thus a great improvement on the crude antigen which consists of ground leprosy nodule and which is difficult to standardize.
- (2) The results are obtained in 24 hours instead of three weeks or more—a great advantage to both the patient and the investigator.
- (3) Undesirable late reactions, ulceration, etc., not uncommonly seen with the classical Mitsuda test are avoided—a very great advantage to the patient.

It is therefore suggested that for doing skin tests in leprosy the isolated antigen can, with great advantage, replace the ordinary lepromin of Mitsuda.

#### *The anomalies of the Mitsuda reaction*

As already mentioned in the previous paper the Mitsuda test has three marked anomalies, namely (a) its lateness, (b) the positive results in non-contacts and (c) the negative results in cases of lepromatous type. We will here discuss these three anomalies in the light of the present work.

(a) *The lateness of the Mitsuda reaction*—In the preceding article it was suggested that the lateness of the reaction was caused by the nature of the material injected, most of the antigen not being free at first, but being liberated gradually in minute amounts over a prolonged period. Histological examination of the nodules produced by injection of ordinary lepromin in patients supported this theory, intact and acid-fast bacilli could still be found several weeks after the injection. This observation is moreover in accordance with the previous

experience that Hansen's bacillus is extraordinarily resistant, for after injection into laboratory animals it can retain its form and acid-fastness for a year or more

Further support was given to the theory by the finding that breaking down of the bacilli enhances the early reaction and reduces the late reaction. The final proof of the truth of the theory is provided by the work on the fractionation of the bacillus reported in the preceding article, the definitely antigenic fraction producing only an early reaction and no late reaction.

It appears, therefore, that it is the constant liberation of minute amounts of antigen, reaching its height three weeks or more after the injection, that causes the characteristic late nodular reaction of the Mitsuda test.

(b) *Positive results in non-contacts*—As already stated positive results to the Mitsuda antigen in non-contacts have been reported by several workers and confirmed by Dharmendra and Jaikaria (1941). No satisfactory explanation has yet been given and the present work has not so far explained this phenomenon. Our work does, however, give some ground for the hope that an explanation may be forthcoming. The bacillary protein has been divided into three fractions, all antigenic, and it may be that one of the fractions is specific. If this is so, a specific allergic skin reaction will be made available.

At one time before the work had reached its present stage we formulated a hypothesis which was capable of explaining both the lateness of the Mitsuda reaction and the positive results seen in non-contacts. This hypothesis we have now abandoned as untenable and it is mentioned here largely because another leprosy worker (Wade, 1941) has recently advanced a very similar hypothesis. We thought that non-contacts were possibly not allergic at the time of the injection of lepromin, but being potentially allergic might be sensitized and rendered allergic by the antigen liberated in the first few days after the injection and that, later, their tissues might react allergically to the antigen still being liberated at the site of injection. This idea therefore means that the same dose of lepromin might both induce allergy and demonstrate allergy by the nodular reaction three weeks later.

If this theory had been true the soluble antigen should have given negative results in non-contacts. As already reported (Lowe and Dharmendra *loc cit*) such results were not obtained. In 24 of the 39 healthy adults living in circumstances which made it highly improbable that they have ever had contact with cases of leprosy, positive results were seen within 24 hours on testing with the soluble antigen. This evidence showed that the hypothesis mentioned is untenable.

(c) *Negative results in cases of lepromatous type*—The present work has no direct bearing on, and suggests no explanation of, this last anomaly, since there is little indication that the 'lepromatous' cases, while not reacting to ordinary lepromin, will react to isolated protein.

We will briefly outline two main lines of thought bearing on this matter. Firstly, the lack of the response of the tissues in these cases is associated with a heavy bacillary infection and may be similar to the negative tuberculin test seen in very advanced cases of tuberculosis. Secondly, this lack of activity may be inherent in the tissues, and not causally related to the presence of leprosy bacilli in the body.

According to the first view, heavy or repeated infections would tend to break down or undermine the resistance of the body, causing the lepromin reaction to be negative and leading to the development of the lepromatous type of the disease. We have found some relation between the presence of the leprosy bacilli in the lesions and the results of the lepromin test. Even in neural cases, the finding of bacilli in the lesions is very often associated with a weaker reaction than would be seen in similar but bacteriologically negative cases. Cochrane *et al* (1941) have found that in children the proportion of the positive lepromin reactions appeared to be lower in those children who had had closer contact. In the opinion of these authors 'the most important single factor in breaking down cellular resistance in leprosy is continuous contact with an open case'.



## THE IMMUNOLOGICAL SKIN TEST IN LEPROSY

### Part III

#### THE ISOLATED PROTEIN ANTIGEN IN RELATION TO THE ANTIGENS USED BY OTHER WORKERS

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[Received for publication, September 25, 1941]

In the two preceding articles [Parts I and II of this series (Dharmendra, 1942, Dharmendra and Lowe, 1942)] the reactions to lepromin (suspension of ground leprous nodules) to filtrate from it, and to the protein antigen isolated from the leprosy bacilli have been discussed. A mention has been made of some other preparations which have been used by different workers mainly with a view to elucidating the mechanism of the Mitsuda reaction. In the present article it is proposed to discuss the bearing of some of these findings on the present work.

#### *Reactions to disintegrated bacilli*

Nagai (1938) found that keeping leprous nodules over a prolonged period in 5 to 10 per cent lecithin or boiling the nodules in the same solution for half an hour resulted in the loss of acid-fastness of the leprosy bacilli and in their degeneration and granulation. Intradermal injections of a suspension of the nodule boiled in lecithin gave rise to reactions similar to those produced by ordinary lepromin.

Thus, this work gave no additional information regarding the mechanism of the test

Kitano and Inoue (1941) broke down the bacilli by physical, instead of chemical, methods. These workers treated ordinary lepromin with ultra-supersonic waves to break the bacilli contained in it. The lepromin thus treated was found to produce stronger early but weaker late reactions than ordinary lepromin. The filtrate from this treated material was found to give early reactions stronger than those produced by filtrate of ordinary lepromin and no late reactions at all. These workers attribute the early reactions to the dissolved components of the bacilli. They concluded that the soluble elements are unable to produce the Mitsuda reaction which depends on the presence of solid bacillary elements in the injected material. These findings agree with the present findings that breaking down of the bacilli is accompanied by the liberation of larger amounts of soluble antigen, and that this soluble antigen produces an early reaction only. These workers do not appear, however, to have realized the significance of the enhanced early reaction produced by the breaking of bacilli.

*Reaction to the different fractions of the lepromatous nodule*—No other workers have reported on the isolation of chemical fractions of the bacilli themselves. A few workers have, however, attempted to isolate the antigenic fraction or fractions from emulsions made by grinding up lepromatous tissue.

Villela (1938) and Rabello, Thiers-Pinto and Villela (1938) at the Cairo Congress reported the isolation from lepromatous tissues of an active non-lipoid fraction. On injection into patients this fraction produced a reaction similar to that produced by ordinary lepromin. Rabello (1938), Rabello and Villela (1938) and Rabello, Villela and Tostes (1939) re-state the same findings. Villela (1939) has described the method of the preparation of the active fraction, 'apparently of protein nature'. The present findings agree with the findings of these workers to the extent that the lipoid fractions of the nodule are inactive, and that activity is confined to the non-lipoid fraction. The reaction to the active substance of protein nature separated by these workers is, however, of the 'delayed' type, like the classical Mitsuda reaction. This protein fraction isolated from leproma emulsion, therefore differs markedly from the fraction isolated as here reported from the bacilli themselves, which gives only an early reaction of the 'tuberculin' type and no late reaction at all.

It is believed that these workers could not have actually separated the protein fraction of the bacilli, and were really working with unbroken and incompletely broken bacilli together with the proteins from the tissues. This belief receives support from the account given of the method employed by these workers to obtain their non-lipoid fraction. 'Ground leproma, boiled in distilled water, is extracted with petroleum ether and an ether-alcohol mixture is added to the aqueous phase. The precipitate forms the non-lipoid fraction'. Our experience indicates that such treatment is not likely to break up the bacilli and liberate bacillary protein, for the leprosy bacilli are very resistant to chemical agents.

Paras (1938) isolated the major lipoid components (phosphatide, acetone-soluble fat and wax) of lepromatous nodules (the isolation of the non-lipoid fractions

has not yet been reported on) Skin tests on a few cases of leprosy showed that, of these lipid fractions, only the wax produced definite reactions similar to, but not as intense as, those produced by ordinary lepromin. The biological activity of the wax separated by Paras can, it is believed, be explained by assuming that the wax contained some lepra bacilli, and this idea also is supported by his account of his methods. The ground leprosy tissue, after having been treated with alcohol-ether mixture, was macerated with chloroform, and the chloroform with the dissolved wax was separated from the tissue by filtration through a Buchner funnel. The great affinity possessed by chloroform for the bacilli has been mentioned in the first article of this series and it is quite conceivable that some bacilli were carried with the chloroform and were present in the wax obtained by evaporating the filtrate.

*Reaction to a protein isolated from leprosy spleen*—Henderson (1940) isolated proteins from leprosy spleens rich in acid-fast bacilli, by grinding the dried spleen in a ball mill at  $-70^{\circ}\text{C}$  and by extracting the ground material with distilled water or with phosphate buffer. The work was undertaken in the hope of obtaining specific proteins of the leprosy bacillus, which could be used in serological or skin tests for the diagnosis of leprosy. The Joint Committee on Leprosy Skin Tests (1940) used these preparations for making skin tests on (i) bacteriologically positive cases of leprosy, (ii) children of leprosy parents (contact group) and (iii) children of healthy parents, with no history of exposure to leprosy infection (control group). 0.05 mg of the isolated protein in 0.1 c.c. was used for the injection. No late reactions of 'Mitsuda' type were seen. Early (24 to 48 hours) reactions of 'tuberculin' type were seen in some persons in all the three groups. These reactions were, however, very weak, usually consisting of an oedematous area of less than 10 mm. The incidence of positive early reactions in the cases, contacts and non-contacts was 4, 19 and 11 per cent respectively. The Committee concluded that the protein extracts of leprosy spleens 'do not contain any substance to which persons suffering from active leprosy (bacteriologically positive cases), or previously exposed to infection by leprosy, react specifically'. The Committee has explained this lack of reaction as follows —

'Either the material, although derived from spleens rich in acid-fast bacilli, did not contain enough specific protein to elicit a positive reaction in the doses used, or sensitiveness comparable to the tuberculin-sensitiveness of tuberculosis does not exist in leprosy patients of the kind used or in leprosy contacts.'

The antigen used by the Committee and the results obtained appear to show certain similarities to and certain marked differences from the antigen used by us, and from the results obtained by us. We will discuss these similarities and differences.

The reaction to the protein isolated by Henderson from leprosy spleens was similar in type, although seen so rarely, to the reaction to the protein isolated by us from leprosy bacilli, since both the preparations produce an early reaction only.



All the cases tested by the Commission were bacteriologically positive and most appear to have been of the 'lepromatous' type. The fact that slight reactions were produced in only 4 per cent of the cases seems to point to another feature common to the two preparations—the inability to elicit reaction in the 'lepromatous' cases.

On the data available it is impossible to make a comparison of the results in the neural cases. In contacts the incidence and the degree of reaction to Henderson's protein is much less than to the protein isolated by us.

The two preparations appear to differ very markedly in potency. 0.05 mg of the protein prepared by Henderson produced weak and evanescent reactions only (usually less than 10 mm), whereas 0.02 mg of the protein prepared from the bacilli produced strong reactions, the area of erythematous swelling being usually more than 20 mm, often reaching 40 mm, and the reaction persisting for a week or more. This difference in the potency appears to be caused by the difference in the source of the two preparations. Henderson isolated protein from the whole spleen, the product being a mixture of protein from the splenic tissue and the leprosy bacilli. The protein isolated by us was prepared from the bacilli freed from tissue and thus consists of bacillary protein only.

As already stated the Joint Committee has put forward two alternative explanations of the absence of reactions to Henderson's protein both in the cases of leprosy and in contacts. According to the Committee either the material injected did not contain sufficient specific protein or 'sensitiveness comparable to the tuberculin sensitiveness of tuberculosis' does not exist in the type of cases tested and in contacts. We have shown that such sensitiveness does exist in contacts and in cases of the neural type but not in cases of the lepromatous type. In the contacts and in the neural cases (if any) the absence of reaction was, therefore, probably caused by the insufficient amounts of antigen. In the lepromatous cases, which constitute most, if not all, of the cases tested by them, the other factor, lack of sensitiveness also operates, and is the chief factor since even large doses of antigen will not induce reaction in them. The two explanations given by the Committee are thus not truly alternative explanations, neither will explain *all* the observations, a part of them being explained by one and a part by the other.

*Reaction to a substance isolated from the urine of cases of leprosy*—Berny and Mauze (1940) have isolated a substance from the urine of bacteriologically positive 'lepromatous' cases. They consider skin reactions to this substance of diagnostic value. A papule exceeding 1 cm in diameter, and accompanied by erythema and pain, is reported to appear 24 hours after the injection. Positive results have been reported in all the 199 cases of leprosy tested, none of the 91 healthy persons tested showing a positive reaction. We have attempted to confirm the findings of Berny and Mauze. A substance of proteose nature has been isolated from urine of bacteriologically positive lepromatous cases. The reaction produced by intradermal injections of this substance appears to be similar to that produced by other proteoses having nothing to do with leprosy and to be different from the reaction produced by the antigen isolated from the leprosy bacillus. This matter is being studied further.

*Reaction to antigens prepared from cultures of acid-fast bacilli*—Apart from preparations obtained from human leprosy material, several preparations from other sources have been used for doing skin tests in leprosy. These preparations include proteins isolated from cultures of various acid-fast bacteria including some of the supposed cultures of *Mycobacterium leprae* and *Mycobacterium leprae muris*, and various chemical fractions of a supposed culture of *Mycobacterium leprae*.

In the present state of our knowledge it seems to us that no useful purpose will be served by analysing and discussing the findings. In order to assess the significance of the findings made with these preparations it is essential to make a comparative study of the antigenic properties of the different protein fractions, prepared by identical methods, from the Hansen's bacillus and the other acid-fast bacilli. This has not so far been done. A study of this matter is now being undertaken.

#### SUMMARY

1 The findings of other workers regarding the antigenic activity of lepromin treated by various methods and the bearing of these findings on the present work have been discussed.

2 The finding of some workers that the breaking down of the bacilli by physical means is accompanied by an increase in the amount of free antigen in lepromin is in accordance with the present findings.

3 The reports of other workers on fractionation of the leprous nodule (not the bacillus itself) are discussed. It is considered that the reaction produced indicated that the active non-lipoid fraction obtained by Villela and co-workers could not possibly have been a protein antigen. Their results are such as would be expected, and have been obtained by us, with unbroken or incompletely broken bacilli. The methods used by them would, it is believed, neither break down the bacilli nor liberate the antigen.

4 Only one worker has isolated protein by grinding leprous tissue (spleen) rich in acid-fast bacilli. The isolated protein produced only very slight early reactions of the 'tuberculin' type in a few cases and in some contacts. In producing an early reaction only, it resembles the protein isolated from the Hansen's bacillus by us, but it is of very much weaker potency, being a mixture of proteins from splenic tissue and from the bacilli.

5 A proteose isolated from the urine of leprous patients by the methods of Berny and Mauze has produced reaction different in nature from that produced by bacillary antigen.

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## A FLUORIMETRIC METHOD FOR THE ESTIMATION OF RIBOFLAVIN IN FOODSTUFFS

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[Received for publication, September 30, 1941]

SINCE the importance of riboflavin in human nutrition is well established the development of a simple method for estimating this vitamin in foodstuffs is a matter of great importance. Many methods have already been described, some biological and others physico-chemical. The consensus of opinion is that riboflavin values obtained by biological methods are more reliable than those obtained by physico-chemical methods and that the latter cannot, at present, be considered wholly satisfactory either because they are not specific for riboflavin or because all the riboflavin present is not estimated (McCollum *et al*, 1939). Methods may be broadly classified as follows —

### A Biological methods —

- 1 By the growth of young rats (Bourquin and Sherman, 1931, el Sadr, Macrae and Work, 1940)
- 2 By the growth of *Lactobacillus casei* (Snell and Strong, 1939, Fraser, Topping and Isbell, 1940, Strong, Feeney, Moore and Parsons, 1941)

### B Physico-chemical methods —

- 1 Colorimetric estimation of the yellowish green colour of the extract obtained after various methods of purification (Koscharka, 1934, Emmerie, 1938, Wilson and Roy, 1938)
- 2 Determination of the fluorescence of the extracts obtained by various methods of purification by a fluorimeter, colorimeter or photometer (von Euler and Adler, 1934, Murthy, 1937, Wilson and Roy, *loc cit*, Sullivan and Norris, 1939, Hodson and Norris, 1939)
- 3 Determination of the yellowish green colour or of the fluorescence of lumiflavin formed by the irradiation of riboflavin in an alkaline medium (Warburg and Christian, 1933, Kuhn *et al*, 1934, Shaw, 1939, Wilson and Roy, *loc cit*)

A 1 In the biological method, using the growth of rats as the criterion, the provision of a basal diet free from riboflavin but containing all other essential nutrients has until recently not been possible, and most basal diets were deficient in at least one factor of the vitamin B<sub>2</sub> complex besides riboflavin. Carlsson and Sherman (1938) pointed out the possibility of a deficiency of other members of the vitamin B<sub>2</sub> complex in the Bourquin-Sherman diet and suggested that food products, such as liver, kidney, muscle and milk, may contain unknown

growth-promoting factors which are absent from the basal diet. Hence any growth response obtained by the addition of a foodstuff to these diets may be due not only to the supplementary effect of riboflavin, but also to other factors present in the food. Such methods of estimation tend to give too high values for riboflavin. Recently, el Sadr, Macrae and Work (*loc cit*) have suggested an improvement in the rat-growth method by employing a liver or yeast extract treated with Norit charcoal which presumably contained all the known factors of the vitamin B<sub>2</sub> complex, except riboflavin, in place of the alcoholic extract of whole wheat used by Bourquin and Sherman (*loc cit*).

2 Snell and Strong (*loc cit*) used the growth-promoting action of riboflavin on *Lactobacillus casei* as the basis of a test. The method is highly sensitive, but it is probable that certain foodstuffs, e.g. liver, muscle, kidney and milk, may contain certain unknown growth factors for the bacillus not present in the synthetic medium, so that the values obtained might again tend to be too high. Further, certain foodstuffs may contain substances which have an inhibiting effect on the growth of bacteria.

*B* Existing physico-chemical methods are in general limited in value, because, when applied to certain food products, they are either not specific for riboflavin or else all the riboflavin present in a food is not estimated. Ellinger (1938), who has made a critical study of methods, pointed out three possible sources of error in physico-chemical methods, incomplete extraction of the riboflavin present, losses occurring during the various processes of purification, interference caused by the presence of varying amounts of coloured and fluorescent impurities in the purified extracts.

In colorimetric estimation, it is necessary to remove all other pigments before a determination of the riboflavin is possible. Van Eekelen and Emmerie (1935) found, however, that the complete elimination of other pigments was not attained. Estimation of the fluorescence of riboflavin in purified extracts of biological materials by a fluorimeter may give rise to faulty results, since varying amounts of colouring matter and blue fluorescent material are always present in such extracts, with the possible exception of the extract obtained from milk. Recently, Hodson and Norris (*loc cit*) have claimed that the blue fluorescent substances present can be eliminated selectively, by reducing with stannous chloride and sodium hydrosulphite. Repeated attempts by the author to use their technique have not proved successful, since it was found that the interfering blue fluorescent materials behaved in the same manner as riboflavin, when acted upon by the above reagents.

The third method of estimation, i.e. conversion of lactoflavin into lumiflavin and determining the fluorescence of the latter, has in general been considered as an improvement over the colorimetric and fluorimetric methods. Interfering fluorescent substances are, however, present in certain foods, extracted along with lumiflavin by chloroform. Further, the conversion of riboflavin into lumiflavin is not quantitative and the losses depend on the quantity of riboflavin present,

these being greater when the quantity present is small. Losses vary from 13 to 58 per cent, as calculated by Kuhn and his co-workers (*loc cit*)

Since chemical methods are less time-consuming than biological methods and more convenient for the study of the physiological and biological rôle of vitamins, a re-investigation of fluorimetric methods has been carried out, as the result of which a new and simple fluorimetric method has been developed

#### PRINCIPLE OF THE METHOD

The method depends upon the following properties of riboflavin (1) It fluoresces green when exposed to ultra-violet light, and the intensity of the fluorescence can be measured with a photo-electric cell (2) It is not destroyed by mild oxidation in the cold with dilute potassium permanganate, whereas interfering colouring matter present is to a great extent removed (3) The fluorescence due to riboflavin can be completely destroyed by heating the vitamin in N/10 NaOH in a boiling water-bath for 40 minutes, while the blue fluorescent interfering materials, associated with riboflavin in purified extracts of biological materials, are stable under the same conditions

The method is an indirect one. This is due to the fact that the colouring matter present interferes with the accuracy of the method by adsorbing part of the activating and fluorescent light and thus causes the results to be too low. The blue fluorescent substances, on the other hand, interfere with accuracy in the opposite direction. The interfering colouring matter can be removed to a great extent by a preliminary adsorption of riboflavin on Fuller's earth, followed by oxidation in the cold with dilute potassium permanganate solution. The interfering blue fluorescent substances can be differentiated from riboflavin by the fact that their fluorescence remains unaffected after treatment with hot N/10 NaOH, while the fluorescence due to riboflavin is destroyed completely. The difficulty of getting rid of traces of stable interfering pigments is overcome by adding a known amount of a standard solution of riboflavin to the unknown solution after a preliminary measurement with the fluorimeter and determining the extent by which these interfering pigments decrease the value due to the added riboflavin. Any loss of riboflavin occurring during the whole process of extraction and purification can be determined by adding a known amount of standard solution of riboflavin to a known amount of the foodstuff under investigation and estimating the percentage recovery of the added riboflavin. Thus, by applying the three important correction factors, viz (1) the interference due to the alkali-stable fluorescence, (2) the interference due to the stable pigments and (3) the loss, if any, occurring during the process of extraction and purification, the amount of riboflavin can be accurately determined.

Riboflavin is photo-sensitive and ordinary room light may have some destructive effect. It is therefore necessary that the experimental procedure should be carried in a subdued light and solutions kept in a dark cupboard when not being used. These precautions were observed throughout the present work.

## EXPERIMENTAL

*Reagents required —*

- 1 Standard riboflavin (strong) 1 ml = 50  $\mu$ g (Half a ml of pure chloroform is added to 500 c.c. of the standard solution as a preservative. The solution is kept in a brown bottle in a refrigerator. It is advisable to prepare a fresh standard once in every 3 months.)
- 2 Standard riboflavin (dilute) 1 c.c. = 5  $\mu$ g prepared fresh as required by diluting 1 ml of solution (1) to 10 ml with water
- 3 Sulphuric acid 10 N (approx.)
- 4 Sodium hydroxide 10 N (approx.)
- 5 Acetic acid 10 N (approx.)
- 6 Sodium acetate solution 4 N (approx.)
- 7 Acetate buffer pH 5
- 8 Potassium permanganate 3 per cent aqueous solution
- 9 Hydrogen peroxide solution (12 vol.)

*Apparatus* —The fluorimeter consisted of a Weston Photronic cell (Model 594) connected to a Reflecting Galvanometer (Tinslay & Co., London) through a universal shunt. The light source was a Hanovia mercury vapour lamp, with a blue glass-filter. For quantities of 1  $\mu$ g to 10  $\mu$ g of riboflavin, in 11 ml of solution, the galvanometer response was proportional to the riboflavin present and there was no appreciable difference in the intensity of fluorescence with change of pH from 5 to 7, when acetate buffer was employed. The response for 1  $\mu$ g of riboflavin varied from 9 to 12 scale divisions, depending upon the intensity of the lamp. Hence, the fluorimeter was always checked against the standard solution of riboflavin before and after a reading with an unknown solution was taken.

*Procedure* —The method finally adopted, after many preliminary trials, included the following steps —

- (1) Extraction of the food material three times with hot N/20  $\text{H}_2\text{SO}_4$
- (2) Removal of colouring matter by the addition of a slight excess of N lead acetate dissolved in N acetic acid solution. (This step is unnecessary with many foods containing small amounts of colouring matter.)
- (3) Adsorption of the riboflavin present on Fuller's earth (B D H)
- (4) Elution of riboflavin using 50 per cent aqueous alcohol, containing sufficient sodium hydroxide to make a concentration of N/4
- (5) Destruction of the riboflavin present in one half of the eluate, by treatment with N/10 NaOH at 100°C in a water-bath
- (6) Decolorization of the eluates by oxidation in the cold with dilute  $\text{KMnO}_4$  and the excess of  $\text{KMnO}_4$  with hydrogen peroxide. Adjustment of pH to 5 and making up to a known volume
- (7) Estimation of the riboflavin present by a fluorimeter, applying the necessary corrections

A convenient quantity (2 g to 100 g) of the finely powdered or mixed material, containing 30  $\mu$ g to 100  $\mu$ g of riboflavin, was placed in a beaker. Two hundred ml to 500 ml of N/20  $\text{H}_2\text{SO}_4$  were then added (0.5 ml of 10 N  $\text{H}_2\text{SO}_4$  was added to

every 100 ml of water used), the amount of solvent used depending on the bulk of the material. The mixture was constantly stirred and heated in a water-bath maintained at about 70°C to 75°C for 20 minutes. It was then allowed to cool and centrifuged. The residue was extracted again twice in the above manner. If the combined extracts were highly coloured, as in the case of certain vegetables and fruits, slight excess of N lead acetate in N acetic acid solution was added till precipitation was complete. (The clear supernatant layer should not give any precipitate with lead acetate solution.) The precipitate was removed on the centrifuge and washed once with 200 ml of water containing 20 ml of N lead acetate in N acetic acid. (This step is unnecessary with the majority of foods, which contain only small amounts of colouring matter.)

Two g of Fuller's earth (B D H) were then added to the clear centrifugate (if the centrifugate is not clear, it must be filtered through No. 1 Whatman filter-paper) and the mixture shaken vigorously for 5 minutes. It was then transferred to a tall beaker and allowed to stand for 15 minutes. The clear, or fairly clear, supernatant liquid was transferred carefully into the shaking bottle, leaving the activated clay in the beaker, and adsorption repeated as before. The two lots of activated earth were combined, removed on the centrifuge, and washed once with 100 ml of N/10 acetic acid.

Sixty ml of 50 per cent aqueous alcohol and 2 ml of 10 N NaOH were then added to the activated earth in the centrifuge tube, the mixture well stirred with a thick glass-rod, and shaken vigorously for 3 minutes, by closing the mouth with a rubber-stopper. A variety of eluants was tried, but none was found to be as effective and cheap as 50 per cent aqueous alcohol containing sodium hydroxide. The mixture was then centrifuged at high speed for 5 minutes and the clear centrifugate transferred immediately to a conical flask containing 10 ml of 10 N acetic acid. The residue in the tubes was again eluted twice in the above manner, using 0.5 ml of 10 N NaOH instead of 2 ml. The whole process of elution should be carried out as quickly as possible and each lot of eluate should be acidified as described immediately. Riboflavin is slowly destroyed in the cold in prolonged contact with sodium hydroxide. Recovery of added riboflavin has shown that there is practically no loss at this stage under the above conditions. The combined eluates (about 180 ml) were divided into two equal portions ( $T_1$  and  $B_1$ ) for further treatment as described below —

One half ( $B_1$ ) of the eluates was transferred to a 250 ml beaker, first adjusted to pH 10 by the addition of 10 N NaOH (using phenolphthalein as internal indicator) and made alkaline, so that the final concentration of alkali in the solution was about N/20 (0.5 ml 10 N NaOH is added for every 100 ml of solution adjusted to pH 10). The mixture was heated in a boiling water-bath for 40 minutes, to destroy the riboflavin present. This resulted in the concentration of the solution to about 30 ml. The solution was then allowed to cool and acidified by the addition of 4 ml of 10 N acetic acid. Sufficient 50 per cent aqueous alcohol was then added to restore the bulk to the original volume.

Five ml of 3 per cent  $KMnO_4$  were now added to each of the solutions ( $T_1$  and  $B_1$ ) and the mixtures allowed to stand for one minute. Ten ml of hydrogen



peroxide solution (12 vol) were then added to each to decolorize the excess of permanganate present. Fifteen ml and 5 ml of 4 N sodium acetate solution were next added to the solutions  $T_1$  and  $B_1$  respectively, so that the ratio of concentration of sodium acetate to acetic acid was 7 : 3. The solutions, which now have a pH of 5, are filtered and made up to volume (150 to 200 ml). The solution  $T_1$  containing the total fluorescence due to riboflavin and other interfering fluorescent materials may be called the 'test solution', and the solution  $B_1$ , which contains only the fluorescence of the interfering fluorescent substances, the 'blank solution'. Ten ml portions were used for determining the amount of fluorescence present. An exactly similar procedure was followed with the same quantity of foodstuff plus added riboflavin ( $50 \mu\text{g}$ ), the final 'test' and 'blank' solutions obtained being designated  $T_2$  and  $B_2$  respectively.

*Determination of the riboflavin present*—The optical cell containing 11 ml of acetate buffer (pH 5) was first placed and a reading ( $a$ ) was taken. One ml ( $5 \mu\text{g}$ ) of standard riboflavin was then taken in another optical cell, 10 ml of acetate buffer (pH 5) added and a second reading ( $b$ ) taken. Next, 10 ml of each of the solutions,  $T_1$ ,  $B_1$ ,  $T_2$  and  $B_2$  were successively pipetted out into an optical cell, 1 ml of water added to each and readings  $t_1$ ,  $b_1$ ,  $t_2$  and  $b_2$  were taken. Then 1 ml ( $5 \mu\text{g}$ ) of standard riboflavin solution was pipetted out into each optical cell containing 10 ml of solutions  $T_1$ ,  $B_1$ ,  $T_2$  and  $B_2$  and the readings  $t_1 + 5$ ,  $b_1 + 5$ ,  $t_2 + 5$ , and  $b_2 + 5$  were taken.

Let the amount of food taken be  $x \text{ g}$  and the final volumes of  $T_1$ ,  $B_1$ ,  $T_2$  and  $B_2$  be  $y \text{ ml}$  each, and the amount of riboflavin added for determining recovery be  $r \mu\text{g}$ .

#### Calculation —

- 1 The reading due to 11 ml of acetate buffer (pH 5) =  $a$  divisions
- 2 The reading due to  $5 \mu\text{g}$  of riboflavin in 11 ml of acetate buffer =  $b$  divisions
- 3 the reading due to  $5 \mu\text{g}$  riboflavin above =  $b - a$  scale divisions  
and the reading due to  $1 \mu\text{g}$  of riboflavin =  $\frac{b - a}{5}$  divisions
- 4 The reading due to 10 c.c. of sol  $T_1$  =  $t_1$  divisions
- 5 The reading due to 10 c.c. of sol  $T_1$   
after correction for adsorption =  $\frac{t_1 (b - a)}{t_1 + 5 - t_1}$  scale divisions
- 6 The reading due to 10 ml of sol  $B_1$  =  $b_1$  scale divisions
- 7 The reading due to 10 ml of sol  $B_1$   
after correction for adsorption =  $\frac{(b_1 (b - a))}{b_1 + 5 - b_1}$  scale divisions

- 8 the corrected reading due to riboflavin present in 10 ml of sol T<sub>1</sub>

$$= \frac{t_1 (b-a)}{t_1 + 5 - t_1} - \frac{b_1 (b-a)}{b_1 + 5 - b_1} \text{ scale divisions}$$

$$= (b-a) \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\} \text{ scale divisions}$$

- 9 The corrected reading due to riboflavin present in y ml of sol T, corresponding to  $\frac{x}{2}$  g of food

$$= \left( \frac{y}{10} \right) \left( \frac{b-a}{1} \right) \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\} \text{ scale divisions}$$

- 10 The corrected reading due to riboflavin present in 1 g of food

$$= \left( \frac{y}{10} \times \frac{2}{x} \right) \left( \frac{b-a}{1} \right) \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\} \text{ scale divisions}$$

- 11 riboflavin present in 1 g of food before correction for recovery of added riboflavin

$$= \frac{y}{10} \times \frac{2}{x} \times \frac{5}{b-a} \times \frac{b-a}{1} \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\}$$

$$= \left( \frac{y}{x} \right) \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\} \mu g$$

The formula for calculating the percentage of recovery, which can be calculated in a similar manner using the values for  $t_2$ ,  $t_2 + 5$ ,  $b_2$ , and  $b_2 + 5$ , is as follows —

- 12 The quantity of riboflavin found in x g of food

$$= y \left\{ \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right\} \mu g$$

- 13 The quantity of riboflavin found in x g of food + r  $\mu g$  of added riboflavin

$$= y \left( \frac{t_2}{t_2 + 5 - t_2} - \frac{b_2}{b_2 + 5 - b_2} \right) \mu g$$

- 14 recovery of added riboflavin

$$= y \left\{ \left( \frac{t_2}{t_2 + 5 - t_2} - \frac{b_2}{b_2 + 5 - b_2} \right) - \left( \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right) \right\}$$

Recovery per cent

$$= \frac{100y}{r} \left\{ \left( \frac{t_2}{t_2 + 5 - t_2} - \frac{b_2}{b_2 + 5 - b_2} \right) - \left( \frac{t_1}{t_1 + 5 - t_1} - \frac{b_1}{b_1 + 5 - b_1} \right) \right\}$$

The value obtained for the riboflavin content of 1 g of test material as calculated according to formula 11 should be corrected for the recovery found by formula 14

The calculation is illustrated by the example given in the *Appendix*

*Recovery of riboflavin added to foodstuffs*—To known amounts of various foodstuffs, 50  $\mu\text{g}$  of riboflavin were added and the procedure described above followed. The recovery was good in all cases, ranging from 80 to 90 per cent. The results are shown in Table I —

TABLE I  
*Recovery of riboflavin added to foods*

Experiment number	Quantity of foodstuffs	Riboflavin added, $\mu\text{g}$	Total riboflavin found, $\mu\text{g}$	Recovery, per cent
1	Red gram ( <i>Cajanus indicus</i> ), 40 g		88	
	Red gram ( <i>Cajanus indicus</i> ), 40 g	50	133	90
2	Yeast, dried, brewer's, 2 g		111	
	Yeast, dried, brewer's, 2 g	50	154	86
3	Rice polishings, raw, 20 g		60	
	Rice polishings, raw, 20 g	50	108	96

*Total fluorescence and the fluorescence due to riboflavin in purified extracts of foods*—All the foods examined contained varying amounts of blue or greenish-blue fluorescent materials, which were found to be stable to treatment with N/10 NaOH at 100°C for 40 minutes. Riboflavin, on the other hand, completely loses its greenish-yellow fluorescence under the same conditions. In Table II are given the values obtained in terms of riboflavin for the total fluorescence and the fluorescence due to riboflavin present in purified extracts of foodstuffs. It will be evident that the latter constitutes only a fraction of the total fluorescence present in food extracts. For example, black pepper, which contains considerable amounts of an alkali-stable greenish-blue fluorescent material, contains only traces of riboflavin. Murthy (*loc cit*), who followed a procedure in which riboflavin was estimated by simply determining the total fluorescence of purified extracts, reported a high figure of 152  $\mu\text{g}$  per g for the riboflavin content of black pepper. Shaw and Hind (1941), using a modification of the lumiflavin method of Warburg and Christian (*loc cit*) and Kuhn and his co-workers (*loc cit*), reported that the fluorescent material in pepper behaved differently from riboflavin, in that the former was soluble in chloroform, while the latter was not. The lumiflavin method, in spite of its limitations, differentiates between riboflavin and other fluorescent

interfering pigments The author's findings are in accordance with the results of these workers

TABLE II

*Total fluorescence and the fluorescence due to riboflavin  
in purified food extracts*

Name of foodstuff	Total fluorescent materials present in the extract expressed as $\mu\text{g}$ riboflavin per g of food.	Riboflavin present, $\mu\text{g/g}$
Rice polishings, raw	11.2	3.0
Ragi ( <i>Eleusine coracana</i> )	2.0	0.5
Raw milled rice	1.0	0.3
Dried yeast, brewer's	76.0	55.5
Red gram ( <i>Cajanus indicus</i> )	6.0	2.2
Black pepper	54.3	0.7

## RESULTS

The method described was applied to 12 common foods, viz 4 cereals, 2 legumes, 2 vegetables, 2 fruits, rice polishings and 3 samples of dried yeast. The results are shown in Table III. A sample of dried brewer's yeast was found to be a good source of riboflavin, containing 55.6  $\mu\text{g}$  per gramme. Another sample of dried yeast, grown in the laboratory on a synthetic medium containing molasses, gave the high value of 64.8  $\mu\text{g}$  per g, while a sample of dried distillery yeast contained only 27.5  $\mu\text{g}$  per g. Of the four cereals investigated, whole wheat contained 1.2  $\mu\text{g}$ , raw milled rice 0.3  $\mu\text{g}$ , parboiled milled rice 0.8  $\mu\text{g}$ , and ragi 0.5  $\mu\text{g}$  per g respectively. Raw rice polishings contained only 3  $\mu\text{g}$  per g. These results are in contrast with the results obtained with other members of the vitamin B complex, viz B<sub>1</sub>, nicotinic acid, pyridoxin (B<sub>6</sub>), which have been found to be present in fair amounts in whole cereals, and in greater amounts in raw rice polishings (Aykroyd, Krishnan, Passmore and Sundararajan, 1940, Aykroyd and Swaminathan, 1940, Swaminathan, 1940, 1941). The two pulses investigated contained appreciable amounts of riboflavin, while vegetables and fruits are poor sources. The results reported appear to correspond well with various biological and fluorimetric data available in the literature (Wilson and Roy, *loc cit*, Hodson and Norris *loc cit*, Boas Fixsen and Roscoe, 1940, Lanford, Finkelstein and Sherman, 1941).

The riboflavin value of whole milk can be taken as about 1.5 micrograms per c.c., as estimated directly in trichloroacetic acid filtrates. Fluorescent substances other than riboflavin are present in milk in negligible quantities. In relation to its content of solids, milk is among the richest sources of riboflavin.

TABLE III

*Riboflavin content of certain foodstuffs.*

Name of foodstuff	Botanical name	Riboflavin, $\mu\text{g/g}$
Rice, raw, milled		0.3
Rice, parboiled, milled		0.8
Rice polishings, raw		3.0
Ragi	<i>Eleusine coracana</i>	0.5
Whole wheat		1.2
Red gram	<i>Cajanus indicus</i>	2.5
Black gram	<i>Phaseolus mungo</i>	1.8
Black pepper	<i>Piper nigrum</i>	0.7
Cabbage	<i>Brassica oleracea capitata</i>	0.5
Carrot	<i>Daucus carota</i>	0.3
Orange	<i>Citrus aurantium</i>	0.4
Papaya	<i>Carica papaya</i>	0.3
Tree tomato	<i>Cyphomandra betacea</i>	0.4
Yeast, dried, brewer's		55.5
Yeast, dried ( <i>Torula</i> yeast, grown on medium containing molasses)		64.8
Yeast, dried, distillery		27.5

## DISCUSSION

The method should be suitable for application to a wide range of materials containing varying amounts of riboflavin. It was found possible with practice to assay 4 foods in 2 days. The extraction procedure was chosen after considerable trial had been made of procedures already described by other workers, who used aqueous or acid alcohol or aqueous acetone. It was found to give better results in that the extraction was more complete, the extract, at the same time, being less contaminated with interfering pigments and other fluorescent substances. As

regards specificity, it appears to compare well with the lumiflavin method of Warburg and Christian (*loc cit*) and Kuhn *et al* (*loc cit*). Further, it has two distinct advantages over the lumiflavin method. Firstly, it is less laborious, and, secondly the loss of riboflavin occurring in the method is small in comparison with the heavy inconstant losses of 13 to 50 per cent occurring in the lumiflavin method (Kuhn *et al*, *loc cit*).

### SUMMARY

1 A fluorimetric method for estimating the riboflavin content of foodstuffs is described

2 The various procedures and corrections necessary to overcome the four main difficulties due to (a) incomplete extraction, (b) interference from stable pigments, (c) interference from fluorescent materials and (d) losses occurring during the process of purification of the extracts are described

3 The riboflavin content of 12 representative foods, viz 4 cereals, 2 pulses, 2 vegetables, 2 fruits, rice polishings and 3 samples of dried yeast, has been determined. Dried yeast is a rich source. The other foods were generally poor in the vitamin, pulses and whole cereals being somewhat superior to the others. The values obtained appear to correspond well with the results of biological assays reported in the literature.

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## APPENDIX.

*Example of calculations followed with a sample of Cayanus indicus*

1 Red gram 40 g (for direct determination)	2 Red gram 40 g + 50 $\mu$ g riboflavin (for recovery test)
The vol of 'test' sol T <sub>1</sub> , corresponding to 20 g red gram = 200 ml	The vol of 'test' sol T <sub>2</sub> , corresponding to 20 g red gram + 25 $\mu$ g riboflavin = 200 ml
The vol of 'blank' sol B <sub>1</sub> , corresponding to 20 g red gram = 200 ml	The vol of 'blank' sol B <sub>2</sub> , corresponding to 20 g red gram + 25 $\mu$ g riboflavin = 200 ml

Serial number	Volume of solution tested, ml.	Reading of the scale	Calculation for correction	Corrected reading of the scale	Reading due to riboflavin present in 10 ml extracts
1	Acetate buffer (pH 5) 11 ml.	7 (a)			
2	Standard riboflavin 5 $\mu$ g in 11 ml buffer	53 (b)		46 (standard)	
3	Ten ml. sol. T <sub>1</sub> + 1 ml of water	44 (t <sub>1</sub> )	$\frac{(b-a)}{t_1 + 5 - 1} \quad 10 \frac{53-7}{81-44}$	54.7 (test 1)	18.9
4	Ten ml sol T <sub>1</sub> + 5 $\mu$ g riboflavin	81 (t <sub>1</sub> + 5)			
5	Ten ml sol B <sub>1</sub> + 1 ml of water	28 (b <sub>1</sub> )	$\frac{b_1 (b-a)}{b_1 + 5 - b_1} \quad 10 \frac{28 \times 46}{64-28}$	35.8 (blank 1)	
6	Ten ml sol B <sub>1</sub> + 5 $\mu$ g riboflavin	64 (b <sub>1</sub> + 5)			
7	Ten ml sol T <sub>2</sub> + 1 ml. of water	51 (t <sub>2</sub> )	$\frac{t_2 (b-a)}{t_2 + 5 - t_2} \quad 10 \frac{51 \times 46}{84-51}$	71.1 (test 2)	28.0
8	Ten ml sol T <sub>2</sub> + 5 $\mu$ g riboflavin	84 (t <sub>2</sub> + 5)			
9	Ten ml. sol B <sub>2</sub> + 1 ml of water	30 (b <sub>2</sub> )	$\frac{b_2 (b-a)}{b_2 + 5 - b_2} \quad 10 \frac{30 \times 46}{62-30}$	43.1 (blank 2)	
10	Ten ml. sol B <sub>2</sub> + 5 $\mu$ g riboflavin	62 (b <sub>2</sub> + 5)			

The reading due to the riboflavin present in 10 ml of sol T = 18.9 divisions

The reading due to the riboflavin present in 200 ml of sol T<sub>1</sub> = 20 × 18.9

Riboflavin present in 200 ml of sol T<sub>1</sub>, corresponding to 20 g =  $\frac{20 \times 18.9}{9.2}$  μg

Riboflavin present in 1 g of red gram =  $\frac{20 \times 18.9}{9.2 \times 20}$  = 2.0 μg

The reading due to riboflavin present in 10 ml of sol T<sub>2</sub> = 28 divisions

The reading due to riboflavin present in 200 ml of sol T<sub>2</sub>, corresponding to 20 g red gram + 25 μg riboflavin = 20 × 28 divisions

Riboflavin found in 20 g red gram + 25 μg added riboflavin =  $\frac{20 \times 28}{9.2}$  μg

Recovery of added 25 μg riboflavin =  $\frac{20 \times 28}{9.2} - \frac{20 \times 18.9}{9.2}$  = 19.8 μg

Recovery per cent =  $\frac{19.8 \times 100}{25}$  = 79.2

Riboflavin in 1 g of red gram after correction for recovery =  $\frac{2.0 \times 100}{79.2}$  = 2.5 μg

The values obtained above for the riboflavin present in 1 g red gram, and the percentage of recovery, can be obtained from the formula given in a previous page as follows —

1 Riboflavin found in 1 g red gram before correction for recovery

$$= \left( \frac{y}{x} \right) \left\{ \frac{t_1}{t_1 + \bar{s} - t_1} - \frac{b_1}{b_1 + \bar{s} - b_1} \right\} \mu\text{g} = \left( \frac{200}{40} \right) \left\{ \frac{44}{81 - 44} - \frac{28}{64 - 28} \right\} = 2.1 \mu\text{g}$$

2 Recovery of added riboflavin per cent

$$= \frac{100y}{r} \left\{ \left( \frac{t_2}{t_2 + \bar{s} - t_2} - \frac{b_2}{b_2 + \bar{s} - b_2} \right) - \left( \frac{t_1}{t_1 + \bar{s} - t_1} - \frac{b_1}{b_1 + \bar{s} - b_1} \right) \right\}$$

$$\frac{100 \times 200}{25} \left\{ \left( \frac{51}{84 - 51} - \frac{30}{62 - 30} \right) - \left( \frac{44}{81 - 44} - \frac{28}{64 - 28} \right) \right\} = 79.5 \%$$

3 Riboflavin present in 1 g red gram =  $\frac{2.1 \times 100}{79.5}$  = 2.6 μg





## FLUORIMETRIC ESTIMATION OF RIBOFLAVIN IN URINE

BY

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[Received for publication, September 30, 1941]

STUDIES on the urinary excretion of riboflavin by human beings have already been reported by Helmer (1937) and Emmerie (1936). Emmerie used a colorimetric method on purified extracts of urine which involved the employment of a photometer, while Helmer used a biological rat-growth technique. Riboflavin is an essential food factor for human beings (Sebrell and Butler, 1938, 1939, Sydenstricker, Sebrell, Cleckley and Kruse, 1940, Kruse, Sydenstricker, Sebrell and Cleckley, 1940) and studies on the metabolism of the vitamin are greatly facilitated by the existence of a simple test for detecting it in urine. In the present paper a fluorimetric method suitable for this purpose is described. Some observations on the urinary excretion of riboflavin by a normal individual, before and after the ingestion of varying quantities of pure riboflavin, are also included. During the progress of this work Strong *et al* (1941) and Sebrell and his co-workers (1941), using the bacterial method of Snell and Strong (1939), have reported results on the excretion of riboflavin by human beings.

### PRINCIPLES OF THE METHOD

These are essentially the same as those of the method described for foodstuffs (Swaminathan, 1942). Urines and extracts of urines purified by the usual procedures of adsorption on Fuller's earth, elution and decolorization, contain varying amounts of colouring matter and blue fluorescent substances which interfere with the colorimetric or fluorimetric estimation of the riboflavin present. The same procedures have been adopted to allow for the interference caused by these substances. The reagents needed are the same as those required for the determination of riboflavin in foods.

*Procedure.*—Twenty five ml. portions of 24 hour specimens of normal urine, or urine collected after test doses, were measured out into 4 small conical flasks (100 ml. capacity) labelled T<sub>1</sub>, B<sub>1</sub> and T<sub>2</sub>, B<sub>2</sub>. Standard riboflavin (5 µg or 25 µg) was then added to the urine in the flasks T<sub>1</sub> and B<sub>1</sub> to find the percentage recovery. (Usually for 25 ml. portions of normal urine and urine collected after test doses, 5 µg and 25 µg of riboflavin were added respectively.) The urines in flasks B<sub>1</sub> and B<sub>2</sub> were adjusted to pH 10 (phenolphthalein as internal indicator) by the careful addition of 10 N NaOH and the quantity of NaOH required was noted. After the addition of a further 0.7 ml. of 10 N NaOH to each flask, the flasks were heated in a boiling water bath for 40 minutes, to destroy the riboflavin.

present The contents of the flasks B<sub>1</sub> and B<sub>2</sub> were allowed to cool Three ml of 10 N acetic acid were then added to all the 4 flasks, T<sub>1</sub>, B<sub>1</sub>, T<sub>2</sub> and B<sub>2</sub>. The total quantity of NaOH originally added to flasks B<sub>1</sub> and B<sub>2</sub> was now added to flasks T<sub>1</sub> and T<sub>2</sub>, so as to make the ratio of the concentration of sodium acetate acetic acid the same in all cases Four ml. each of 3 per cent KMnO<sub>4</sub> were added to all the 4 flasks to oxidize the colouring matter present After one minute, 8 ml of hydrogen peroxide were added to each flask, to decolorize the excess of KMnO<sub>4</sub> present Sufficient sodium acetate solution (4 N) was then added to all the flasks so that the ratio of the concentration of sodium acetate acetic acid present was 7 : 3 and pH about 5 The solutions were then filtered and made up to volume (50 ml to 200 ml), so that the quantity of riboflavin present in 10 ml of solution may be 1 µg to 5 µg Ten ml portions of the extracts T<sub>1</sub>, B<sub>1</sub>, T<sub>2</sub> and B<sub>2</sub> were used for determining the quantity of fluorescence present

If the concentration of riboflavin is less than 1 µg in 1 ml of urine, the procedure described in the previous paper (Swaminathan, 1942) for food extracts should be followed, as the quantity of riboflavin present is too small to be accurately estimated by the direct method described in this paper

*Determination of riboflavin* —Essentially the same methods of estimation and calculation as those employed for foodstuffs were followed The calculations made in the case of one sample of urine are given as an example —

*Example*

- (1) Volume of 24-hour specimen of normal human urine = 2,500 ml
- (2) Volume of urine measured out into the flasks T<sub>1</sub>, B<sub>1</sub>, T<sub>2</sub> and B<sub>2</sub> respectively = 25 ml
- (3) Quantity of riboflavin added to flasks T<sub>2</sub> and B<sub>2</sub> for finding the percentage recovery = 5 µg
- (4) Final volume of the solutions T<sub>1</sub>, B<sub>1</sub>, T<sub>2</sub> and B<sub>2</sub> respectively was 50 ml

Solution T<sub>1</sub> represents the 'test' containing the fluorescence due to riboflavin and interfering substances present in 25 ml of urine

Solution B<sub>1</sub> represents the 'blank' containing the fluorescence due to the interfering substances alone, present in 25 ml of urine

Similarly, solutions T<sub>2</sub> and B<sub>2</sub> represent the 'test' and 'blank' respectively, the former containing the fluorescence due to the riboflavin and interfering substance present in 25 ml of urine + 5 µg riboflavin, and the latter containing the fluorescence due to the interfering substances alone present in 25 ml of urine + 5 µg riboflavin

*Readings and calculation*

Solution tested ml	Reading of the scale divisions	Calculation for correction (scale divisions)	Corrected readings (scale divisions)	Readings due to the riboflavin present in the solutions (scale divisions)
1 Acetate buffer 11 ml	7			
2 Standard riboflavin 5 µg + 10 ml of acetate buffer	57	57-7	50	

## Readings and calculation—concl'd

Solution tested ml	Reading of the scale divisions	Calculation for correction (scale divisions)	Corrected readings (scale divisions)	Readings due to the riboflavin present in the solutions (scale divisions)
3 Ten ml. sol B <sub>1</sub> + 1 ml. of water	22.5	$\frac{22.5 \times 50}{50 - 22.5}$	41	
4. Ten ml. sol. B <sub>1</sub> + 5 µg riboflavin	50			
5 Ten ml. of sol. T <sub>1</sub> + 1 ml. of water	34	$\frac{34 \times 50}{70 - 34}$	47.2	6.2
6 Ten ml of sol. T <sub>1</sub> + 5 µg riboflavin	70			
7 Ten ml. of sol. B <sub>2</sub> + 1 ml of water	23	$\frac{23 \times 50}{52 - 23}$	40	
8 Ten ml. of sol B <sub>2</sub> + 5 µg riboflavin	52			
9 Ten ml. of sol. T <sub>2</sub> + 1 ml. of water	39	$\frac{39 \times 50}{73 - 39}$	57.3	17.3
10 Ten ml of sol. T <sub>2</sub> + 5 µg riboflavin	73			

The quantity of riboflavin present in 10 ml of sol T<sub>1</sub> =  $\frac{6.2}{10}$  µg

The quantity of riboflavin present in 50 ml of sol T<sub>1</sub> corresponding 25 ml of urine =  $\frac{5 \times 6.2}{10} = 3.1$  µg

The quantity of riboflavin present in 2,500 ml of urine corresponding to 24 hours =  $100 \times 3.1 = 310$  µg

The quantity of riboflavin present in 50 ml. of sol T<sub>2</sub>, corresponding to 25 ml. of urine + 5 µg of riboflavin =  $\frac{17.3 \times 5}{10} = 8.7$  µg

Recovery of added riboflavin =  $5.6$  µg = 112 per cent

The recovery test can legitimately be omitted when the procedure is applied to a series of materials of a similar nature, which have been found to give good and constant recovery values

It is essential that the calculation be made as shown above, making the corrections for (a) the adsorption effect of stable interfering pigments, (b) the interference due to other blue fluorescent materials and (c) the loss of riboflavin, if any, occurring during the process, as found by the percentage recovery of added riboflavin

*Recovery of riboflavin added to urine*—Different known amounts of riboflavin were added to 25 ml portions from 24-hour specimens of normal urine and urine collected after test dose, and the above procedure followed. The recovery was good in all cases, ranging from 85 to 106 per cent (Table I)

TABLE I

*Recovery of riboflavin added to urine*

Experiment number	Urine with and without added riboflavin	Total riboflavin found, $\mu$ g	Recovery, per cent
1	25 ml (normal)	3.4	106
	25 ml + 5 $\mu$ g riboflavin	8.7	
2	25 ml (normal)	3.6	92
	25 ml + 5 $\mu$ g riboflavin	8.2	
3	25 ml (test dose 1 mg)	12.7	89
	25 ml + 25 $\mu$ g riboflavin	34.9	
4	25 ml (test dose 2 mg)	18.2	97
	25 ml + 25 $\mu$ g riboflavin	42.5	
5	25 ml (test dose 5 mg)	41.3	93
	25 ml + 25 $\mu$ g riboflavin	64.5	
6	25 ml (test dose 10 mg)	79.6	85
	25 ml + 25 $\mu$ g riboflavin	100.8	

*The excretion of riboflavin by human beings*—The present investigation was carried out on a laboratory worker, consuming a fairly well-balanced diet based on milled rice and including a daily allowance of 20 oz of milk, and fair amounts

of pulse and vegetables The normal daily intake of riboflavin from such a diet was probably 1.2 mg to 1.5 mg

Preliminary study of the urinary excretion of riboflavin (Table II) at two intervals in the 24-hour period, after test doses of 2 mg to 5 mg, showed that about three-fifths of the additional excretion which occurred as a result of the test dose in 24 hours took place in the first 7 hours, and about two-fifths in the second 17-hour period The increase in excretion due to the test dose was complete in 24 hours, as is shown by the return to normal on the sixth day after the test dose had been discontinued The test doses were taken between 9 a.m. and 9.30 a.m.

TABLE II

*Urinary excretion of riboflavin estimated at intervals, before and after test doses*

Consecutive experimental days	Dose of riboflavin, $\mu\text{g}$	URINARY OUTPUT IN 24 HOURS			Increase in excretion due to test dose, $\mu\text{g}$
		1st 7 hour period, 9.30 a.m. — 4.30 p.m., $\mu\text{g}$	2nd 17 hour period, 4.30 p.m. — 9.30 a.m., $\mu\text{g}$	Total 24 hours, $\mu\text{g}$	
1	Nil	77	258	335	
2	Nil	72	243	315	
3	2,000	1,036	702	1,738	1,413
4	2,000	1,115	659	1,774	1,449
5	5,000	2,478	1,702	4,180	3,755
6	Nil	84	266	350	

A further experiment was carried out as follows Twenty-four-hour specimens of urines were collected, usually from 9.30 a.m. to 9.30 a.m. Fifty ml of glacial acetic acid and 2 ml of toluene were placed in the bottles used for the collection of urine, as preservative The specimens were kept in a dark place

The test doses were taken at 9-30 in the morning. The maximum dose of riboflavin was 10 mg. The results are given in Table III —

TABLE III  
*Urinary excretion of riboflavin before and after test doses  
in a normal subject*

Experimental days	Test dose of riboflavin, $\mu\text{g}$	Urinary excretion in 24 hours, $\mu\text{g}$	INCREASED EXCRETION DUE TO THE TEST DOSE	
			$\mu\text{g}$	Per cent
1	<i>Nil</i>	350		
2	<i>Nil</i>	320		
3	1,000	1,196	861	86
4	2,000	1,926	1,591	80
5	5,000	4,295	3,960	80
6	10,000	9,096	8,734	87
7	1,000	1,388	1,053	105
8	1,000	1,214	879	88
9	<i>Nil</i>	344		

#### DISCUSSION OF RESULTS.

The results given in Table I show that the normal daily excretion of riboflavin was about 320  $\mu\text{g}$  to 350  $\mu\text{g}$  when the intake of riboflavin from the diet was of the order of 1.2 mg to 1.5 mg. It appears that about 25 to 30 per cent of the ingested riboflavin was excreted. But after the ingestion of test doses of 1 mg to 10 mg, about 80 to 85 per cent of the test dose was excreted in 24 hours. These results are in contrast with those found in similar studies with vitamin B<sub>1</sub>, nicotinic acid and pyridoxin (vitamin B<sub>6</sub>) (Harris and Leong, 1936, Swaminathan, 1939, 1941). The urinary excretion after the ingestion of test doses of these substances was of the order of 5 to 12 per cent. After the ingestion of 17 mg of riboflavin in

three days, the organism seemed to be 'saturated', as the ingestion of 1 mg of riboflavin on the fourth day resulted in an excretion of a little over 1 mg. It appears likely that a 'saturation' test for the diagnosis of 'partial' deficiency of riboflavin occurring in human beings, similar to that in existence for vitamin C, could be developed. The results described in this paper correspond well with those reported by Strong *et al* (*loc cit*) and Sebrell and his co-workers (*loc cit*), using the bacterial method of riboflavin assay. The rapid excretion of riboflavin is of importance in connection with the dosage employed for therapeutic purposes. It may, however, be that more riboflavin will be retained when it is given to subjects suffering from deficiency of this vitamin. This problem is being investigated. Other workers in the Laboratories have found that certain ophthalmic conditions respond immediately and dramatically to treatment by injection of riboflavin, but tend to recur within a few weeks after treatment is discontinued and the patient returns to his usual diet.

The fluorimetric method for the estimation of riboflavin in urine described in this paper is quite simple, with an accuracy of 10 to 15 per cent. It should prove to be of value in studies on the metabolism of riboflavin in human beings and experimental animals. With practice, 4 to 6 estimations can be carried out in a day by a single worker. The method is much simpler and more specific than the colorimetric method of Emmerie (*loc cit*).

### SUMMARY

A fluorimetric method has been applied for the estimation of riboflavin in urine. The excretion of riboflavin in a normal subject before and after test doses has been determined. The normal daily (24 hours) urinary output ranged from 320  $\mu$ g to 360  $\mu$ g. After the ingestion of test doses of 1 mg to 10 mg of riboflavin orally, about 80 to 85 per cent of the test dose was excreted in the following 24 hours.

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## FLUORIMETRIC ASSAY OF RIBOFLAVIN IN THE URINE AND TISSUES OF RATS

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[Received for publication, September 30, 1941]

THE importance of riboflavin in the nutrition of the rat has been recognized for some years. Rats fed on a diet deficient in this factor cease to grow (Edgar *et al*, 1937) and later develop various pathological lesions (Day *et al*, 1938, Bessy and Wolbach, 1939, el Sadr, 1940). In two previous communications (Swaminathan, 1942*a*, 1942*b*) fluorimetric methods for the estimation of riboflavin in foodstuffs and human urine were described. In the present investigation the test has been used to estimate riboflavin in the urine and tissues of rats fed on diets containing varying amounts of riboflavin.

### EXPERIMENTAL

Four groups of young rats (6 in each group) were kept individually in metabolism cages and fed respectively on the diets shown in Table I. Groups I and II received diet A and groups III and IV diet B. Each rat in groups II and IV received in addition 50  $\mu$ g of riboflavin daily mixed with the diet.

The rats received weighed amounts of the diets, given only in slight excess of food requirements, and records of food intake were maintained. The experiment lasted for a period of 8 weeks. Urine was collected daily. To prevent decomposition of urine, 2 ml of glacial acetic acid were placed in each flask. The metabolism cages, funnels and separators were washed down daily with small quantities of water and the washings mixed with the urine.

*Preparation of the rice polishings concentrate*—Rice polishings (100 g) were extracted twice with 600 ml and 400 ml of hot N/100 sulphuric acid. The extract was adjusted to pH 6 by the addition of barium hydroxide solution. Slight excess of basic lead acetate solution (N) was added to precipitate completely the protein derivatives, colouring matter and riboflavin. Basic lead acetate at pH 7 removes 80 to 90 per cent of the riboflavin in the extract. Excess of lead in the filtrate

## RIBOFLAVIN CONTENT OF TISSUES

*Method of extraction*—Preliminary experiments showed that a hot aqueous or aqueous acid extract of animal tissues contained all the riboflavin present in the tissue with only small amounts of interfering blue fluorescent material, and that the interference caused by these materials was at the most 10 per cent of the total value obtained

A weighed amount (1 g to 5 g) of the finely minced tissue, containing from 10  $\mu$ g. to 30  $\mu$ g riboflavin, was finely ground with glass powder in a porcelain mortar. The pasty mass was transferred to a beaker with 40 ml of hot water and the mixture heated in a boiling water bath for 15 minutes. To this 0.4 ml of 10 N  $\text{H}_2\text{SO}_4$  was added and the heating continued for another 15 minutes. Five ml of 25 per cent trichloroacetic acid containing 0.4 ml of 10 N NaOH were then added, the mixture allowed to cool and centrifuged. The clear centrifugate was separated and the residue extracted twice in the above manner, using half the quantity of the solvent and reagents. The combined centrifugates (amounting to about 90 ml) were adjusted to pH 7, filtered and made up to 100 ml. The riboflavin in 10 ml portions of the extract was estimated directly in a fluorimeter, allowance being made for the 'water blank' and for the interference caused by adsorption of the activating and fluorescent light by the colouring matter present. The recovery of riboflavin added to the same amount of the tissues was also determined. The method of calculating the riboflavin present is illustrated by an example given below. The recovery of added riboflavin was good. The values obtained for the riboflavin content of tissues of rats from different groups are given in Table IV

*Example* —

- 1 Weight of liver taken = 2.0 g
- 2 Quantity of riboflavin added to determine the recovery = 25  $\mu$ g
- 3 Final volume of the extracts = 250 ml

Let the extracts corresponding to 2 g liver and 2 g liver + 25  $\mu$ g riboflavin be called  $T_1$  and  $T_2$

*Calculation* —

Solution tested	Scale reading	Calculation for correction	Corrected reading	Reading due to riboflavin in extract
11 ml of water	7			
Standard riboflavin 5 $\mu$ g in 11 ml of water	57	57 - 7	50	
Ten ml of sol $T_1$ + 1 ml of water	29	$\frac{29 \times 50}{69 - 29} - 7$	29.3	29.3
Ten ml of sol $T_2$ + 5 $\mu$ g of riboflavin	69			
Ten ml of sol $T_2$ + 1 ml of water	38	$\frac{38 \times 50}{78 - 38} - 7$	40.5	40.5
Ten ml of sol $T_2$ + 5 $\mu$ g of riboflavin	78			
Riboflavin in 10 ml of sol $T_1$ = $\frac{29.3}{10}$ $\mu$ g				

Riboflavin in 250 ml of sol  $T_1$ , equivalent to 2 g of liver =  $\frac{25 \times 29.3}{10} = 73.3$   $\mu$ g

Riboflavin in 1 g of liver = 36.7  $\mu$ g

Riboflavin in 1 ml of sol  $T_2$  =  $\frac{40.5}{10}$   $\mu$ g

Riboflavin in 250 ml of sol  $T_2$  corresponding to 2 g of liver + 25  $\mu$ g riboflavin =  $\frac{25 \times 40.5}{10} = 101.3$   $\mu$ g

Recovery of added riboflavin = 28.0  $\mu$ g = 112 per cent

*Effect of different methods of extraction on the estimation of riboflavin in liver* — During the progress of this work, van Duyne (1941) pointed out that the extraction of riboflavin from tissues is incompletely achieved by the usual methods and that preliminary hydrolysis with pepsin is necessary for complete extraction. He stated that his technique could not be applied to muscle, as the final extracts obtained exhibited a strong blue fluorescence. Ochoa and Rossiter (1939) found that the riboflavin-adenine-nucleotide present in animal tissues was easily extracted with hot water. Snell and Strong (1939), Mickelson *et al* (1939) and Fraser *et al* (1940) autoclaved the finely minced tissues, suspended in water. In the present investigation extraction with hot dilute acid has been employed. In view of the findings of van Duyne (*loc cit*), the conditions necessary for complete extraction of riboflavin from tissues have been re-investigated. Two methods were followed (a) the method described in this paper, applicable only to extracts containing negligible amounts of interfering blue fluorescent materials and (b) the general procedure described before for other biological materials. The results are shown in Table III —

TABLE III

*Effect of different methods of extraction on the estimated riboflavin content of (rat) liver*

Method of extraction *	RIBOFLAVIN $\mu\text{g/g}$	
	†A Method of estimation	†B Method of estimation
1	30.7	28.5
2	33.5	28.8
3	33.5	29.2
4	36.8	29.7
5	45.8	28.5

\* Note on methods of extraction —

- (1) Extraction thrice with hot N/20 HCl and proteins precipitated with trichloroacetic acid after neutralizing with HCl present and the rest of procedure same as under (2)
- (2) The procedure described in the present paper was followed
- (3) The finely minced tissue was heated with 40 ml of N/20 HCl for 30 minutes in a boiling water bath, allowed to cool and incubated at 37°C for 20 hours the rest of the procedure being as under (2)
- (4) The finely minced tissue was suspended in 40 ml of N/20 HCl, 0.5 g pepsin added, heated for 30 minutes in a boiling water bath to inactivate the pepsin added, cooled and incubated at 37°C for 20 hours. The rest of the procedure was as under (2)
- (5) The finely minced tissue was suspended in 40 ml of N/20 HCl, heated in a water bath for 30 minutes and cooled. 0.5 g pepsin was then added and the mixture incubated at 37°C for 20 hours, the rest of the procedure being same as under (2)

† Note on the method of estimation —

METHOD A The method described in the present paper

METHOD B The method of adsorption and decolorization described before for foodstuffs was applied to the trichloroacetic acid extracts obtained.

Table III shows that the values obtained by the first method were in general 10 per cent higher than those obtained by the second method when extraction procedures 1 to 3 were followed and that the tissue extracts contained small amounts of interfering fluorescent material, accounting for 10 per cent of the riboflavin values obtained. After hydrolysis with pepsin, the 'apparent' riboflavin value increased. At the same time, the amount of interfering fluorescent material is increased, the fluorescence due to interfering substances accounting for about 40 per cent of the 'apparent' riboflavin values obtained. Hence it appears quite probable that the increase in 'riboflavin' values observed by van Duyne after hydrolysis of the tissues with pepsin can be ascribed to the fluorescence due to interfering materials liberated during hydrolysis.

*Riboflavin content of tissues*—Values for liver, muscle and heart of the rats in groups I and III were lower than the corresponding values obtained for the same tissues of rats in groups II and IV receiving riboflavin supplements (Table IV). This observation, considered in conjunction with the fact that in groups I and III excretion exceeded intake suggests that the excess of riboflavin excreted was derived from the liver, muscle, heart and possibly other tissues. There was no appreciable difference in the riboflavin content of the kidney in all groups. Since there was no difference between the riboflavin content of the tissues of animals in groups II and IV and that of the tissues of normal young stock rats, it appears that about 55 per cent of ingested riboflavin is used for maintenance and growth.

TABLE IV

*Mean riboflavin content of rat tissues*

( $\mu\text{g/g}$  fresh tissue)

Group number	Diet	Liver	Muscle	Heart	Kidney
I	Basal riboflavin deficient diet	14.4	2.3	16.4	29.5
II	Same diet + 50 $\mu\text{g}$ riboflavin daily per rat	29.2	3.8	26.7	31.3
III	Poor rice eater's diet	21.3	2.7	20.5	32.6
IV	Same diet + 50 $\mu\text{g}$ riboflavin daily per animal	33.3	4.4	26.1	35.7
V	Young rats on stock diet (whole wheat, milk, pulses and vegetables)	33.8	4.2	27.3	34.8

## DISCUSSION

The figures obtained for riboflavin in normal tissues are of the same order as those reported by Fraser *et al* (*loc cit*) and Vivanco (1935), who employed bacterial and fluorimetric methods respectively, and slightly higher than those recorded by Kuhn *et al* (1935). The reduction in the riboflavin content of liver, muscle and heart in the animals on the deficient diet is of the same order as that occurring in the case of vitamin B<sub>6</sub> in rats and nicotinic acid in monkeys (Swaminathan, 1941a, 1941b), when a parallel experimental technique is employed. The results correspond well with those of Fraser *et al* (*loc cit*), who carried out similar experiments using the bacterial method. Recently Ochoa and Rossiter (*loc cit*) have studied the riboflavin-adenine-dinucleotide content of the tissues of normal and riboflavin-deficient rats and obtained values of the same order. The rats kept on riboflavin-deficient diets for 8 weeks continued to excrete small amounts of riboflavin in the urine, a similar observation has been made by Fraser *et al* (*loc cit*). None of the animals showed any signs of riboflavin deficiency during the experimental period.

## SUMMARY

1 Balance experiments were carried out on four groups of rats for a period of 8 weeks. Groups I and II were fed on a riboflavin-deficient basal diet supplying about 0.9  $\mu\text{g}$  per rat, while groups III and IV were fed on a poor rice diet supplying about 2.2  $\mu\text{g}$  riboflavin daily per animal. In addition, each animal in groups II and IV received 50  $\mu\text{g}$  riboflavin daily mixed with the diet.

2 In the groups fed on the unsupplemented diet, the daily excretion of riboflavin per rat was 1.9  $\mu\text{g}$  and 2.6  $\mu\text{g}$  respectively. The rats in groups II and IV receiving riboflavin supplements excreted about 18  $\mu\text{g}$ , i.e. 45 per cent of the ingested riboflavin.

3 The riboflavin content of the liver, muscle and heart of rats fed on the unsupplemented diets was lower than that of the same tissues in the rats receiving additional riboflavin and in stock animals.

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## DIET SURVEYS AND INVESTIGATIONS OF HÆMOGLOBIN LEVELS IN COORG

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[Received for publication, September 30, 1941]

THE small province of Coorg, with a population of between 160 and 170 thousand at the 1931 Census, is situated in South India to the west of Mysore. It is a mountainous tract with extensive coffee plantations, jungle and forest, subject to a heavy annual rainfall. The population is mostly employed on estates or in cultivating small plots of ground in the neighbourhood of villages. Except in one small area, where ragi (*Eleusine coracana*) is grown, the only cereal cultivated is rice. The production of honey for sale is one of the industries of the province, and Coorg honey is known all over India.

Coorg is an unhealthy area, with a high death and infant mortality rate. The mortality in females aged 15 to 40 years is the highest recorded in India. Malaria and hookworm are prevalent and numerous cases of anæmia, debility and malnutrition are seen in the hospitals. In 1940 it was decided to undertake dietary investigations and propaganda work, with the object of improving the state of nutrition of the population. The second author (M S B), formerly attached to the Coorg Public Health Department, attended the 1940 nutrition class in the Nutrition Research Laboratories, and subsequently returned to Coorg to take up work in this field. The present paper briefly records the results of diet surveys in three areas and presents data about hæmoglobin levels in school children and pregnant women. An experiment on the effect of the routine administration of iron on hæmoglobin in school children is also described.

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† Employed under a grant from the I. R. F. A.



## DIET SURVEYS

These were carried out in the principal town, Mercara, and in the villages of Kadagadal and Igodulu, situated 6 and 12 miles from Mercara respectively. The usual methods of the Laboratories were followed (Aykroyd and Krishnan, 1937). Intake of various foods is shown in Table I. The families belonged to the poorer classes and were typical of the majority of the population in the localities in question.

TABLE I

*Intake of foods in ounces per consumption unit daily*

Locality	Mercara	Kadagadal	Igodulu
Number of families	26	25	18
Duration of survey (days)	15	15	15
Season	October	November December	March
Home pounded rice (raw and par-boiled)	21.5	22.0	23.3
Other cereals	0.2	0.7	0.1
Pulses	1.0	0.8	0.7
Leafy vegetables	0.2	0.4	0.5
Other vegetables	5.2	5.1	3.8
Vegetable oil (mostly coconut)	0.3	0.3	0.5
Ghee and butter	0.1	<i>Nil</i>	<i>Nil</i>
Whole milk	2.8	1.0	2.0
Fruit	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>
Meat, fish and eggs	1.6	1.2	0.2
Sugar and jaggery	1.9	0.9	0.9

The diets in all three groups were of the usual type consumed by poor rice eaters in India and in general showed the usual qualitative defects, which have been fully discussed in the *Memor 'The Rice Problem in India'* (Aykroyd, Krishnan, Passmore and Sundararajan, 1940). It is the general habit to consume raw or parboiled rice prepared on a foot-pounder and retaining a high percentage of pericarp. Intake of leafy vegetables is low and that of milk negligible. No fruit was taken.

The population of Coorg is almost entirely non-vegetarian. The Coorgs enjoy the privilege of being allowed to keep guns without permission or licence and are keen hunters. Wild game, including boar, is an occasional article of diet. Pork

is a popular dish and small salted fish obtained from rivers and ponds, eaten whole, are consumed fairly regularly by the poorer classes. The intake of meat and fish by the Mercara families was exceptionally high for India. Throughout the province generally however, the consumption of flesh foods is small, in spite of the predilection of the population for them. Eggs are taken in negligible quantities.

*State of nutrition and deficiency diseases*—School children in the districts surveyed and other village areas were observed to be in a poor state of nutrition. Underweight was very general. Thirty-eight per cent showed phrynoderma, 7 per cent angular stomatitis and 4 per cent xerophthalmia (Bitot's spots).

It is of interest to note that tropical sloughing phagedæna, also known as tropical ulcer, is very common in Coorg. A dozen or so cases are nearly always under treatment in the Mercara Civil Hospital at any given time. Various workers have concluded that malnutrition plays a part in the causation of this condition.

*Anæmia*—Superficial inspection showed many children and adults in villages and plantations to be visibly anæmic. Of 455 children examined in Mercara and neighbouring villages 204 (44.8 per cent) had enlarged spleens. In some rural areas the spleen rate is higher than in Mercara. In Kadagadal, Cherala and Madapur a rate of 79 per cent was recorded.

An investigation of hæmoglobin levels in groups of children was undertaken. This was followed by an experiment of the effect of the administration of iron tablets on hæmoglobin prompted by the desirability of discovering a cheap, feasible and immediately practicable method of improving the condition of children. Hæmoglobin levels in pregnant and non-pregnant women were also studied. A Hellige normal hæmometer with square tubes was used and the results are stated in grammes of Hb per cent.

Children from 5 to 12 years were investigated. Table II shows the average Hb level in a number of school groups.—

TABLE II  
*Hæmoglobin in Coorg school children*

Locality	Number of children	Hb grammes per cent	Standard deviation	Coefficient of variation	
Mercara school	A	96	10.63	$\pm 0.80$	7.5
	B	91	10.58	$\pm 0.85$	8.1
	C	115	10.87	$\pm 0.64$	6.5
Cherala	43	10.04	$\pm 1.33$	13.3	
Kadagadal	50	9.81	$\pm 0.67$	6.8	
Madapur	60	9.67	$\pm 1.16$	12.0	

The mean level was lower in the villages than in Mercara. Average figures were approximately the same with the age groups 5 to 12 and no difference between boys and girls was observed. The values were below those recorded for non-pregnant adults in other parts of India, *vide* the table of normal hæmoglobin levels given by Napier (1940). Hare (1940) gives a mean value of 9.62 for 12 children aged 0 to 9 belonging to coolie families in Assam and 13.0 for 11 children in the same age groups drawn from the families of the Indian staff of tea-estates, i.e. families at a better economic level.

*Administration of iron*—The tablets used were 2.5 grain (0.16 g.) ferrous sulphate tablets, obtained from the London Mission, Neyyoor, Travancore, costing Rs. 1.8-0 per 1,000. They were reported to contain traces of manganese and copper. In the experiment in Mercara 2 tablets daily were given and in a later experiment in Madapur 4 tablets. With the co-operation of the teachers, there was no difficulty in administration. The control group in Madapur received peppermints. The experiment lasted 2 months, the number of days in which iron tablets were given being approximately 40.

In Mercara the experimental group consisted of 96 children in school A, while the controls were 91 children in school B. The children in each case were of similar economic status. In Madapur the groups consisted of 22 and 23 children respectively attending the same school. In both experiments the various ages were equally represented in the two groups. Determinations of Hb were carried out on both groups at the beginning and end of the experimental period. The small differences between the mean levels in experimental and control groups at the beginning of the experimental period were not statistically significant. Records of height and weight and spleen enlargement were also kept. Hæmoglobin readings are given in Table III—

TABLE III

*Hæmoglobin levels before and after the administration of iron (g per cent)*

			Number of children	Enlarged spleen	Average Hb at beginning of experiment	Standard deviation	Average Hb at end of experiment	Standard deviation	Average gain or loss, g
Mercara	School A (experimental group)		96	32	10.63	± 0.80	11.20	± 0.85	+ 0.6
	School B (control)		91	28	10.59	± 0.85	10.4	± 0.79	- 0.2
Madapur	Experimental group		22	18	9.60	± 1.08	10.82	± 0.86	+ 1.1
	Control		23	23	9.70	± 0.96	9.98	± 0.72	+ 0.3

The difference between the mean changes in hæmoglobin values in the experimental and control groups were statistically significant in both cases, being more than twice the standard error of the difference between the means

There appeared to be some tendency for the children in the groups receiving iron to put on more weight than the control groups but owing to the small numbers in the various age groups satisfactory conclusions on this point could not be reached. The administration of iron had no effect on the incidence of vitamin-deficiency diseases

Probably larger doses of iron would have had a greater effect on hæmoglobin levels. This would however, mean increased expense. It was argued that the cost of supplying 4 tablets daily to each child in a school of 50 children would be only a few rupees per month, and that this expenditure, perhaps limited to certain schools in which the children were particularly anæmic, would probably not be beyond the scope of the educational department

#### HÆMOGLOBIN IN PREGNANT WOMEN

Records were taken of hæmoglobin levels in 150 pregnant women attending the Civil Hospital, Mercara. All except 16 of these were 28 weeks pregnant or more and the majority were approaching, or at, full term. The cases were consecutive and not selected for anæmia. The great majority attended hospital for delivery or for advice about pregnancy, and not to seek treatment for anæmia. It is, however, probable that women with severe anæmia come to hospital for childbirth more readily than healthy women, so that the group investigated may not be quite typical of pregnant women in general in the province

The hæmoglobin of a control group of 38 apparently healthy non-pregnant women was also determined. In both groups the majority belonged to the coolie or poor agriculturist classes. The results were as follows —

	Number	Hb, grammes per cent	Standard deviation	Coefficient of variation
Pregnant women	150	8.16	± 2.58	31.6
Non pregnant women	38	10.13	± 0.93	9.13

The differences in the mean levels are statistically significant

Some degrees of anæmia is apparently physiological. Napier (*loc cit*) suggests that a figure below 7.7 g per cent may be regarded as indicating anæmia in pregnant women of the coolie class. This is 27 per cent below the usual average of 10.5 g per cent for women of this class and allows for physiological anæmia and individual variation. Of the 150 pregnant cases 52, or 35.3 per cent gave a hæmoglobin figure below 7.7. In 15 per cent the Hb content of the blood was below 5 g per cent

Cases of anæmia were distributed according to parity as follows —

	Number of cases	Per cent anæmic
Primiparæ	34	14.7
II para	33	48.5
III para	19	52.6
IV para	34	41.2
V to VI para	30	26.6

The percentage of cases of severe anæmia was thus smaller among primiparæ than multiparæ. Here again, however, the distribution of anæmia according to parity in the hospital group may not correspond with that existing in the population in general. For example, healthy primiparæ may be more anxious to be delivered in hospital than healthy multiparæ.

### DISCUSSION

Malaria, hookworm and malnutrition combine to make Coorg province an unhealthy area. The condition of the population as regards anæmia appears to resemble closely that existing in Assam, where detailed investigations have been carried out by Napier and his colleagues, and others. Probably the same picture will be observed in any tropical or sub-tropical plantation area in which blood-destroying diseases are prevalent and the diet deficient.

It is of interest to compare Coorg with the Nilgiri plateau, where there is a large coolie population employed on tea-estates. Malaria and hookworm are for practical purposes non-endemic in the latter area. The type of diet consumed by tea-plantation workers in the Nilgiris has been described by Krishnan (1939). Comparison of the data given by him with the results of the diet surveys recorded in the present paper shows that the Nilgiri diet is actually rather more deficient than the Coorg diet. Anæmia of pregnancy is, however, rare in the Nilgiris (Radhakrishna Rao, 1938). This suggests that poor rice diets normally contain sufficient hæmopoietic substances to prevent serious anæmia and that other factors are necessary to produce anæmia of pregnancy on a wide scale as a formidable public health problem. It may be that poor rice diets contain just enough iron for maintaining the Hb content of the blood at a reasonable level under normal conditions, but that there is no margin of safety to meet increased requirements caused by loss and destruction of red blood corpuscles.

The distribution of iron tablets in an area like Coorg, while it may have some good temporary effect, is essentially a makeshift. What is required is a general effort to raise standards of health, and especially campaigns against malaria, hookworm and malnutrition.

## SUMMARY

1 Diet surveys have been carried out in three areas in Coorg province. The diets showed the usual defects of poor rice diets.

2 Hæmoglobin levels in various groups of school children were investigated. Average values ranged from  $9.67 \pm 1.16$  to  $10.87 \pm 0.64$ . The regular administration of ferrous sulphate tablets increased the hæmoglobin content of the blood.

3 The average Hb level in a group of 150 pregnant women was  $8.16 \pm 2.58$ . Hæmoglobin values were below 7.7 per cent in 35.3 per cent of this group. The average Hb figure for non-pregnant women was  $10.13 \pm 0.93$ .

## ACKNOWLEDGMENT

We are grateful to Captain G. P. Charlewood, I.M.S., Medical Officer of Health and Civil Surgeon, Coorg, for his interest and help in these investigations.

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# THE EFFECT OF MILLING ON THE VITAMIN B<sub>1</sub> (THIAMINE) AND NICOTINIC ACID CONTENT OF INDIAN WHEAT PRODUCTS

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[Received for publication, September 30, 1941]

INDIA produces 9 to 10 million tons of wheat per annum of which 8 to 8½ million tons are consumed in the country. Wheat is the staple grain of North and North-West India and in the country as a whole comes next to rice in extent of cultivation. Until recently there were no roller mills in India and the wheat was converted into flour ('atta') by hand- or power-driven grinding stones and consumed as whole-meal flour. This is still the general practice in rural areas. Roller mills have, however, now been introduced and produce different flour products. The 'atta' (which once meant whole-wheat flour) turned out by such mills contains variable amounts of germ and bran and thus varies in nutritive value. Queries are frequently received in the Laboratories about the vitamin content of different kinds of wheat flour—suji, rawa, atta No 1, etc.—sold in the market. It was found that no definite information was available on this question and the present investigation was undertaken to provide it. The vitamin B<sub>1</sub> and nicotinic acid content of different products have been determined. Both these important food factors are found in greater concentration in the germ and bran than in the endosperm, and the amounts present provide a suitable index of nutritive value.

## PROCESSING OF WHEAT

(1) *In stone milling* the whole grain is simply ground between two stones rotated by hand or by mechanized power. The coarser bran particles are sieved out before use.



(2) *Roller mills* —Here the grains are crushed between several pairs of grooved steel or chilled iron rollers, which are operated by steam or electric power. Each successive pair of rollers is set closer than the preceding one. The grain is gradually disintegrated and pulverized and the endosperm separated from the bran. After each 'break', i.e. passage through a pair of rollers, the crushed grains are sieved through meshes of different grades and refined white flour (maida) and semolina (suji and rawa) are removed. Most flour from roller mills as sold on the market is a mixture of maida, suji, rawa and bran, and different types contain different proportions of these fractions. It is common to use the terms atta No 1, 1½, 1½, 2 and 3, these flours being prepared by the admixture of increasing quantities of coarse bran with the other grades of flour and being sold at decreasing prices. Flours from different mills with a similar grade mark, e.g. atta No 2, may not be precisely similar products. The Report on the Marketing of Wheat in India (1937) points out that the trend of prices for the various products is fairly uniform in different centres, so that in general grade marks convey an idea of the nature of the flour. There are, however, many individual exceptions. These types of flour are cheaper than whiter grades of flour and are much used by the poorer classes.

According to the Report quoted above, there were, in 1937, 80 roller mills in India. It is stated that the wheat requirements of these is approximately one million tons per annum and that of this two-fifths are converted into maida (refined flour). The total output of 'maida' is thus about 400,000 tons per annum.

## RESULTS

Samples of different wheat products were obtained from a large modern roller mill in North India. All these were from the same original batch of wheat. Samples were also prepared in the Laboratories from the same original sample of wheat by grinding in a hand stone-mill and an iron grinder worked by electricity. Vitamin B<sub>1</sub> was determined by the thiochrome method (Aykroyd, Krishnan, Passmore and Sundararajan, 1940). Nicotinic acid was estimated by the cyanogen bromide-aniline method (Swaminathan, 1938). The results are shown in the Table. Values for some other cereals are inserted for purposes of comparison.

## DISCUSSION

The Table shows that most of the wheat products sold in India are fairly rich in vitamin B<sub>1</sub> and nicotinic acid. The practice of grinding in stone-mills, followed in villages and small towns, causes little loss of these vitamins. The coarser grades of flour sold by roller mills, which cater for the needs of big cities and the surrounding areas, are somewhat richer in vitamin B<sub>1</sub> and nicotinic acid than whole wheat, presumably because they contain a greater proportion of germ and bran. It remains the general habit in India to consume wheat in the form of unleavened cakes (chapatties) made from wheat flour which is 'whole' or nearly so.

TABLE

*Vitamin B<sub>1</sub> and nicotinic acid content of wheat products and other cereals*  
(Micrograms per gramme)

Wheat product	Vitamin B <sub>1</sub>	Nicotinic acid	Other cereals	Vitamin B <sub>1</sub>	Nicotinic acid
Whole wheat	4.0	48.0	Whole rice	2.9	45.0
Maida (white flour)	1.2	13.0	Hand pounded raw rice	2.4	24.0
Suji (semolina)	1.5	16.0	Milled raw rice	1.0	16.0
Rawa ( , )	1.3	18.0	Milled parboiled rice	2.2	38.0
Atta No. 1	4.0	40.0	Ragi ( <i>Eleusine coracana</i> )	4.2	14.0
Atta No. 1½	5.0	54.0	Cholam ( <i>Pennisetum typhoides</i> )	3.5	15.0
Atta No. 2	6.0	65.0			
Ground whole wheat from electrically driven steel grinder	4.1	48.0			
Whole wheat (ground by hand in stone grinder)	4.2	50.0			

The 'superior' types of flour, whiter in appearance and less nutritive, are usually consumed by well-to-do Indians, as also by the European and Anglo-Indian communities. In large cities white bread is made from refined flour and its popularity among Indians appears to be on the increase. From the standpoint of nutrition, it is highly desirable that the ancient habit of eating wheat as whole-wheat 'chapatties' should not give ground in favour of a preference for white bread.

Those who prefer bread should eat 'whole meal' rather than white bread. This is of considerable importance in the case of the Anglo-Indian community, many members of which consume what may be described as a 'poor European diet', deficient in 'protective' foods.

Even the most refined grades of white flour produced in India are probably somewhat richer in vitamin B<sub>1</sub> than certain types of 'patent' white flour prepared

and sold in Europe and America Williams and Spies (1938) give a 'preferred value' of 0.5 micrograms of vitamin B<sub>1</sub> per gramme for 'patent' flour, which is less than half that determined for 'maida' in the present investigation

### SUMMARY

The vitamin B<sub>1</sub> and nicotinic acid content of various grades of wheat flour manufactured and sold in India has been determined. Most of the common grades available on the market were found to be good sources of these constituents.

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## HÆMATOLOGICAL INVESTIGATIONS IN SOUTH INDIA

### Part VI

#### THE RED CELL DIAMETER IN HEALTHY EUROPEANS AND INDIANS

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IN a communication from these Laboratories, Sankaran and Radhakrishna Rao (1938) described a relatively simple and rapid method of determining red blood cell diameters. This is a modification of the method described by Hynes and Martin (1936) in which blood films are projected on a screen and the images of the corpuscles measured with a graduated celluloid protractor. The mean red cell diameter determined by this method for normal South Indians, male and female, was  $6.85\mu$ , with a range of  $6.27\mu$  to  $7.38\mu$ . Napier, Sankaran, Swaroop and Narasimha Rao (1939), using the same technique, obtained a higher figure (mean  $7.288\mu$ , range  $6.644\mu$  to  $7.932\mu$ ) for healthy male Bengalee Indians. The South Indian average is also lower than the figure for Europeans given by Price-Jones (1933) (mean  $7.202\mu$ , range  $6.661\mu$  to  $7.492\mu$ ). No satisfactory explanation of this difference could be suggested and further work on the problem has been undertaken. The mean r b c diameter in British soldiers stationed in the Nilgiri hills and in South Indians living on the plains has been studied.

#### METHOD

This is essentially the same as that described by Sankaran and Radhakrishna Rao (*loc cit*). These workers used a Spencer's microscope with an oil-immersion objective 95, tube length 160 mm and a Zeiss eye-piece K 20  $\times$  (compensating eye-piece). This combination of lenses was used in conjunction with the Bausch

and Lomb euscope for projecting blood films on a ground-glass screen, giving a magnification of about 2 000. A description of the apparatus used, the preparation of the celluloid protractor and the technique of measuring the red blood cells, was given in the previous paper. As a rule, corpuscles in the centre of the field are measured, leaving out those at the periphery of the ground-glass screen. This procedure was adopted not only to eliminate spherical aberration, but also to facilitate the manipulation of the celluloid protractor. In the present investigation, the celluloid protractor was graduated to measure to the nearest  $0.25\mu$ .

### SUBJECTS INVESTIGATED

A group of British soldiers, between 18 and 25 years of age, stationed in Wellington (6,000 feet above sea-level) for over a year, was studied. All were healthy and active young men living under good conditions. The second group consisted of 25 individuals both male and female, belonging to the staff of the District Headquarters Hospital, Coimbatore (about 1,350 feet above sea-level). All were healthy South Indians who had never visited the hills, with no history of dysentery, malaria, kala-azar or other chronic infections. Most of them were nurses, midwives and nursing orderlies in the hospital.

Smears were taken from the blood obtained by pricking the finger. All samples were collected in the forenoon. In the Coimbatore series, the hæmoglobin content of the blood was also estimated (by the Hellige hæmometer) to ensure that the persons were not anæmic. The smears were stained with Leishman stain and then counter-stained with 1 per cent aqueous eosin.

### RESULTS

The results are given in Tables I and II —

TABLE I

*Red blood cell diameter in Europeans on the Nilgiris*

Serial number	R b c mean diameter (M in $\mu$ )	Standard deviation ( $\sigma$ in $\mu$ )	Coefficient of variation
1	7.209	0.405	5.617
2	7.253	0.388	5.343
3	7.234	0.410	5.668

Mean diameter of r b c =  $7.135\mu$   
 Range of value of mean diameter =  $6.840\mu$  to  $7.399\mu$   
 Standard deviation =  $0.130\mu$   
 Coefficient of variation = 1.817 per cent,

TABLE I—*concl'd*

Serial number	R b c mean diameter (M in $\mu$ )	Standard deviation ( $\sigma$ in $\mu$ )	Coefficient of variation
4	7 345	0 435	5 916
5	7 399	0 402	5 433
6	7 260	0 387	5 324
7	7 277	0 416	5 721
8	7 130	0 406	5 694
9	7 236	0 363	5 021
10	7 100	0 380	5 350
11	7 143	0 424	5 929
12	7 119	0 388	5 443
13	7 144	0 354	4 955
14	7 056	0 374	5 300
15	7 006	0 402	5 738
16	7 127	0 385	5 395
17	7 269	0 403	5 544
18	7 024	0 353	5 026
19	7 110	0 406	5 710
20	6 840	0 394	5 760
21	7 041	0 392	5 568
22	7 027	0 350	4 974
23	7 098	0 413	5 812
24	6 886	0 323	4 746
25	7 057	0 370	5 244

Mean diameter of r b c = 7 135 $\mu$   
 Range of value of mean diameter = 6 840 $\mu$  to 7 399 $\mu$ .  
 Standard deviation = 0 130 $\mu$   
 Coefficient of variation = 1 817 per cent.

TABLE II

*Red blood cell diameter in Indian subjects in Coimbatore*

Serial number	Sex	Hæmoglobin in grammes per 100 c c blood	R b c mean diameter (M in $\mu$ )	Standard deviation ( $\sigma$ in $\mu$ )	Coefficient of variation
1	Male	17.1	6.956	0.434	6.239
2	"	18.7	7.035	0.319	4.534
3	"	15.7	7.146	0.422	5.905
4	"	15.7	6.980	0.420	6.014
5	"	15.4	7.012	0.421	6.001
6	"	16.3	7.244	0.385	5.312
7	"	17.0	7.178	0.387	5.387
8	"	16.4	6.850	0.374	5.462
9	"	16.4	7.263	0.400	5.501
10	"	15.4	7.086	0.374	5.273
11	"	13.8	7.037	0.375	5.335
12	"	14.0	7.056	0.410	5.815
13	"	17.5	7.053	0.420	5.953
14	Female	13.3	7.188	0.447	6.216
15	"	14.7	7.435	0.417	5.610

Mean diameter of r b c

Range of value of mean diameter

Standard deviation

Coefficient of variation

= 7.079 $\mu$ = 6.781 $\mu$  to 7.435 $\mu$ = 0.139 $\mu$ 

= 1.967 per cent

TABLE II—*conold*

Serial number	Sex	Hæmoglobin in grammes per 100 c c. blood	R b c mean diameter (M in $\mu$ )	Standard deviation ( $\sigma$ in $\mu$ )	Coefficient of variation
16	Female	13.5	6.927	0.286	4.132
17	"	13.3	7.062	0.306	4.330
18	"	12.5	7.052	0.335	4.750
19	"	13.7	7.142	0.380	5.327
20	"	13.5	7.257	0.342	4.713
21	"	13.5	7.052	0.395	5.602
22	"	12.5	6.781	0.331	4.884
23	"	17.1	6.930	0.362	5.222
24	"	14.0	7.082	0.356	5.032
25	"	15.1	7.156	0.339	4.741

Mean diameter of r b c

= 7.079 $\mu$ 

Range of value of mean diameter

= 6.781 $\mu$  to 7.435 $\mu$ 

Standard deviation

= 0.139 $\mu$ 

Coefficient of variation

= 1.967 per cent

The average red blood cell diameter in group I (British soldiers) was found to be 7.135 $\mu$  with a range of 6.840 $\mu$  to 7.399 $\mu$ . In group II (Coimbatore South Indians) the mean red cell diameter was 7.079 $\mu$  with a range of 6.781 $\mu$  to 7.435 $\mu$ . The statistical data obtained in this investigation together with values given by Price-Jones (*loc cit*) and Napier *et al* (1939, 1941) are shown in Table III.

The mean red cell diameter of British soldiers was lower than the figure given by Price-Jones (*loc cit*) for Europeans. This difference in the mean diameter (0.067 $\mu$ ) is statistically *just* significant, being more than twice (0.062), but less than thrice, the standard error of the difference between the two means.



TABLE III.  
*Normal red blood cell diameters.*

Serial number	Authors (year)	Number of cases	Race	Mean diameter (M in $\mu$ )	Maximum diameter in $\mu$	Minimum diameter in $\mu$	Standard deviation in $\mu$	Coefficient of variation
1	Price-Jones (1933)	100	Europeans	7 202	7 492	6 661	0 172	2 3
2	Present authors (1942)	25	British soldiers	7 135	7 399	6 840	0 130	1 817
3	Sankaran and Radhakrishna Rao (1938)	25	South Indians--Nilgiris	6 85	7 38	6 27	0 28	4 1
4	Present authors (1942)	25	South Indians--Coimbatore	7 079	7 435	6 781	0 139	1 967
5	Naper, Sankaran, Swaroop and Narsaiah Rao (1939)	50	Indian Bengalees	7 288	7 932	6 644	0 408	
6	Naper, Sen Gupta and Chandra Sekar (1941)	25	Mainly Bengalees (normal Indians)	7 3438	7 750	6 937	0 1328	1 81

In group II the mean diameter was higher than that recorded by Sankaran and Radhakrishna Rao (*loc cit*) for South Indians living in the Nilgiri hills. It is, however, lower than that given by Napier and his co-workers (1939) for Bengalee Indians. In both instances, the differences in the mean diameters are statistically significant. In the former, the difference in the mean diameter ( $0.229\mu$ ) is 3.663 times the standard error of the difference between the two means (0.06252). In the latter, the difference in the mean diameter ( $0.209\mu$ ) is 4.3 times the standard error of the difference between the two means (0.0486).

### DISCUSSION

The advantages of the method were pointed out in the original communication (Sankaran and Radhakrishna Rao, *loc cit*). Some details of the technique were omitted from this paper, since the authors were closely following Hynes and Martin's original technique. In a recent communication Napier *et al* (1941) have criticized the method on the ground that high magnifications (of the order of 4,200) are not suitable for measuring red cell diameters, owing to the spherical aberration thereby produced. A magnification of this order has not, however, been used in the method followed in the Laboratories. With the apparatus employed, and at the magnification mentioned above, spherical aberration is not a serious interfering factor. The standard deviation and the coefficient of variation of the values given by Sankaran and Radhakrishna Rao are of the same order as those recorded by Napier *et al* (1941), using a similar magnification.

The Indian subjects previously investigated in the Nilgiris (mean diameter  $6.850\mu$ ) and the Coimbatore group (mean diameter  $7.079\mu$ ) were all healthy South Indians living under similar conditions as regards activity and diet. The only obvious environmental difference between the two groups is the altitude at which they were living. It is therefore possible that altitude may have some influence on red cell diameter. The fact that the observed values for British soldiers in Wellington were lower than those given by Price-Jones (*loc cit*) lends support to this view. Very little is, however, known about the effect of altitude on the red cell diameter. The only reference to this question found in the literature available in Coonoor is in a paper by Gulland and Goodall (1914). These authors, referring to the average size of the red corpuscles, remark that 'the size shows very little variation in health. The average size, however, is considerably reduced in the case of the inhabitants of high altitudes'. No other explanation appears to account satisfactorily for the differences observed. The same apparatus and technique have been used throughout the investigations.

### SUMMARY

1. The mean red cell diameter of a group of healthy British soldiers stationed in Wellington (6,000 feet above sea-level) and a group of healthy Indians living in Coimbatore (about 1,350 feet above sea-level), South India, were investigated by Hynes and Martin's method (1936), as modified by Sankaran and Radhakrishna Rao (*loc cit*).

2 The mean diameter of the first group was  $7\ 135\mu$  with a range of  $6\ 840\mu$  to  $7\ 399\mu$ . The corresponding figures in the second group were mean  $7\ 079\mu$ , and range  $6\ 781\mu$  to  $7\ 435\mu$ . These figures are compared with those obtained by other workers.

3 It is suggested that altitude may have some influence on the red cell diameter.

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## BALANCED DIETS

### Part III.

#### THE EFFECT OF PARCHING ON THE BIOLOGICAL VALUE OF THE PROTEINS OF SOME CEREALS AND PULSES

BY

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[Received for publication, August 5, 1941 ]

IN 1917 Osborne and Mendel made the observation that the commercial soya bean cake which had been subjected to high temperatures during the process of expressing the oil promoted better growth in rats than did the raw soya bean. They also noticed an increased food consumption on the diet containing the former ingredient. The authors suggested therefore that the higher nutritive value of soya bean cakes was due to this factor. Hayward, Steenbock and Bohstedt (1936) re-investigated the question utilizing the cakes obtained at different temperatures during the processing of soya bean. They found that the soya bean moistened and pressed between 140°C and 150°C gave a cake possessing a higher nutritive value than the one pressed at lower temperatures. The exposure of the beans to the high temperatures was for the short period of only 2.5 minutes or less. The bean subjected to dry heat at 135°C for 1½ hours did not increase its nutritive

value, on the contrary this procedure decreased it. They further concluded from the metabolic studies that the high nutritive value of soya bean was not due to increased food intake but was due to a better utilization of the soya bean protein. The biological values and digestibility coefficients of high temperature soya bean cake and the raw soya bean were 51 against 41 and 87 against 85.

These investigations therefore suggested that heating the air-dried gram (bean) did probably improve its nutritive value through an improvement in the biological value of its proteins. This observation if confirmed would be of great importance to India, for, in several parts of this country, a variety of pulses and cereals are submitted to more or less common methods of roasting or parching prior to consumption. Apart from the fact that roasting the pulse or cereal obviates the necessity for cooking, the justification of this extensive practice might be sought not only in the improved palatability but probably (according to the popular belief) also in the improved nutritive value of the material thus treated. It was necessary, therefore, to put this belief to the experimental test. Hence, the investigation on the results of parching was undertaken.

#### EXPERIMENTAL

*Methods of parching*—The cereals and pulses were bought locally and parched by a professional according to the methods common to several parts of India. The actual process is somewhat primitive and uncontrolled and may be described as follows. The cereal or pulse to be parched is sprinkled with a little water or with a solution of common salt in water to give saltish taste after parching (which improves the palatability). The cereal or the pulse thus treated is mixed with about four times its own volume of pre-heated sand contained in the frying pan kept on an open fire, the temperature of the sand being about  $235^{\circ}\text{C}$  to  $240^{\circ}\text{C}$ . The cereal or the pulse is then allowed to be parched (or roasted) by rapid mixing in the frying pan by means of a ladle. The temperature of the mixture at which the parching took place was between  $132^{\circ}\text{C}$  and  $136^{\circ}\text{C}$ . The initial temperature of the substance to be parched was somewhere between  $26^{\circ}\text{C}$  and  $29^{\circ}\text{C}$  depending upon the atmospheric temperature. The rise of temperature of the cereal or the pulse from its initial temperature to  $132^{\circ}\text{C}$  to  $136^{\circ}\text{C}$  took place within 2 to 3 minutes. The parched material was then separated from the sand by sieving.

In the case of rice, four parched products are generally obtained according to the previous treatment of it. They are as follows: (1) 'Lahu' (paddy bloomed\*) paddy is parched directly like any other pulse or cereal as described above and then de-hulled. The final product obtained is white and bloomed. (2) 'Pohe' (beaten rice) paddy is steeped in water at a temperature of about  $60^{\circ}\text{C}$  to  $70^{\circ}\text{C}$  and is kept overnight in contact with water during which time it is allowed to cool. The water is then drained off, a small quantity of about one lb. of paddy at a

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\*The product obtained by this process differs in appearance from the product of the process (4). The latter resembles a vesicle and is described as 'puffed' rice. When paddy is parched the rice grain not only swells up but also opens out to a certain extent.

time is put in a frying pan, dried by heating the paddy till it just begins to crack and then beaten in a mortar while hot by means of a wooden pestle (3) 'Murmura' ('pohe' parched) in this case the beaten rice obtained in (2) above is fried in a frying pan till it blooms (4) 'Kurmura' paddy is boiled in water, dried and de-hulled The grain is then parched like any other cereal or pulse till it blooms

### THE DIETS

(a) *Nitrogen-free diet*—A diet which contained practically no nitrogen was prepared by using starch made nitrogen free by repeated extractions with dilute hydrochloric acid, salt mixture prepared according to Osborne and Mendel (1919), cod-liver oil and sugar It was not possible to prepare the diet absolutely free from nitrogen The range of variation of nitrogen content in the nitrogen-free diet was from 0.021 to 0.050 per cent

(b) *Experimental diets*—The experimental diets were prepared by adding the necessary ingredients in the desired proportions as shown in Table I Experiments were carried out on a 5 per cent level of protein intake In all these experiments nitrogen was estimated by Kjeldahl's method

### THE TECHNIQUE

The technique employed was similar to that used by Chick *et al* (1935a, 1935b), Martin and Robison (1922) and Mitchell (1924) This is described briefly below—

A group of six rats (3 males and 3 females) was used Before the first test diet was given the rats received the nitrogen-free diet for seven days The first three days of each period was regarded as preparatory, allowing the rats to accommodate themselves to the new diets The collection of urine and faeces was confined to the remaining four days of each period The rats were given rest for four days after each experimental period with the nitrogen-free diet as well as with the test diets During the rest period they received the stock diet

*Formula used*—The formulæ used by Chick and her co-workers, which have been adopted here, are as follows—

The relative biological value (B V) of a protein (P) is expressed as—

$$100 \times \frac{\text{Body N saved}}{\text{Body N absorbed}} = 100 \left[ 1 - \frac{U_p - U_e}{I_p - (F_p - F_e)} \right]$$

Where  $I_p$ ,  $U_p$  and  $F_p$  are the daily N intake, urinary nitrogen and faecal nitrogen respectively on a diet containing protein and  $U_e$  and  $F_e$  are the daily endogenous N excreted in urine and faeces respectively on a nitrogen-free diet  $F_p - F_e$  represents the nitrogen in the faeces derived from undigested food protein and  $I_p - (F_p - F_e)$  the true nitrogen intake on a diet containing protein The relative digestibility coefficient (D C) of a given protein P is expressed as—

$$100 \times \frac{\text{Food N digested}}{\text{Food N intake}} = 100 \left[ 1 - \frac{F_p - F_e}{I_p} \right]$$

### Composition of diets used (unparched and parched cereals and pulses)

Other quantities in  $g$  are shown in the table

Diets investigated	Botanical name of the foodstuffs investigated	EXPERIMENTAL FOODSTUFFS USED		STARCH ADDED IN		CANE SUGAR ADDED IN		PERCENTAGE OF NITROGEN ON DRY WEIGHT		CRUDE PROTEIN PER CENT ON DRY WEIGHT (N x 6.25)
		Un parched	Parched	Un-parched	Parched	Un parched	Parched	Un-parched	Parched	
<i>Nitrogen-free diet</i>										
1 a	Bengal gram	20 0	20 0	75 0	55 0	10	10	0 021 to 0 050	0 807	5 044
1 b	" "	40 0	40 0	110 0	110 0	20	20	0 050	1 000	10 000
2	Green gram	21 0	20 4	54 0	54 6	10	10	0 874	0 902	5 637
3	Black gram	24 0	23 0	51 0	52 0	10	10	0 837	0 851	5 321
4	Horse gram	24 0	23 0	51 0	52 0	10	10	0 835	0 923	5 772
5	Dried peas	26 0	24 4	49 0	50 6	10	10	0 832	0 846	5 288
6	'Ragi'	72 0	70 0	30	50	10	10	0 909	0 916	5 725
7	'Juar'	54 2	51 7	20 8	23 3	10	10	0 016	0 909	5 083
8	Maize	55 4	40 3	19 6	34 7	10	10	0 896	0 880	5 503
	Paddy parboiled						5		0 888	5 548
	" and parched ('kurmura')						5		0 930	5 812
	Paddy rice, polished	82 0				3		0 902		5 812
	" beaten ('pohe')		80 0				5		0 902	5 635
	" and parched ('murmura')		80 0				5		0 914	5 715
	Paddy parched ('lahi')		80 0				5		0 902	5 635

*Biological value and digestibility coefficient of unparoled and paroled turtles*

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TABLE II

Biological value and digestibility coefficient of unparched and parched pulses

Particulars of the diet	Percentage of N in the diets, dry weights	Number of rats used	Nitrogen intake, mg (average)	Urine N, mg (average)	Faecal N, mg (average)	Food N absorbed, mg (average)	Body N saved, mg (average)	Biological value (average)	Digestibility coefficient (average)
1 a { Nitrogen free diet									
Bengal gram, unparched	0.042	6	2.91	17.36	10.97	53.13	41.48	78.19	85.72
" , parched	0.807	6	64.88	29.01	10.82	53.13	41.48	78.19	85.72
1 b { Nitrogen-free diet									
Bengal gram, unparched	0.807	6	65.90	25.93	18.81	55.82	47.24	84.01	88.69
" , parched	0.042	6	3.17	22.53	13.08	110.32	88.04	74.55	80.09
2 { Nitrogen free diet									
Dried peas, unparched	1.020	6	137.02	52.93	27.45	129.03	101.22	77.97	88.89
" , parched	1.000	6	148.70	51.24	28.61	129.03	101.22	77.97	88.89
3 { Nitrogen free diet									
Green gram, unparched	0.021	6	1.86	18.02	12.64	58.40	40.85	69.53	80.96
" , parched	0.832	6	73.80	30.36	20.19	62.16	48.04	78.18	83.85
4 { Nitrogen free diet									
Green gram, unparched	0.874	6	72.82	40.30	25.48	61.70	35.80	50.04	86.94
" , parched	0.021	6	1.66	20.40	16.00	67.84	47.88	70.21	85.20
5 { Nitrogen free diet									
Black gram, unparched	0.043	1	3.61	7.50	16.71	80.20	51.27	63.85	85.67
" , parched	0.837	1	97.48	36.51	30.40	76.54	50.41	65.85	83.75
6 { Nitrogen free diet									
Horse gram, unparched	0.851	1	94.90	33.68	31.54	50.89	31.20	61.35	77.18
" , parched	0.043	6	4.73	6.25	10.83	50.01	42.12	69.77	79.42
7 { Nitrogen free diet									
Horse gram, unparched	0.895	6	70.71	25.95	26.08	50.01	42.12	69.77	79.42
" , parched	0.023	6	70.44	24.45	25.24	50.01	42.12	69.77	79.42



TABLE II—*concl'd*

Particulars of the diet	Percentage of N in the diets, dry weight	Number of rats used	Nitrogen intake, mg (average)	Urinary N, mg (average)	Faecal N, mg (average)	Food N absorbed, mg (average)	Body N saved, mg (average)	Biological value (average)	Digestibility coefficient (average)
6 { Nitrogen free diet 'Juar', unparched " parched	0.039 0.916 0.909	6 6 6	3.44 77.64 76.94	33.43 43.84 39.44	16.55 23.29 22.06	67.78 68.14	56.37 62.15	83.06 90.88	91.44 92.70
7 { Nitrogen free diet Maize, unparched " parched	0.039 0.896 0.880	4 4 4	2.68 54.77 69.03	24.61 42.17 44.64	14.37 24.76 27.00	41.07 53.76	25.11 34.24	60.14 63.94	80.29 80.83
8 { Nitrogen free diet 'Ragi', unparched " parched	0.050 0.909 0.916	6 6 6	6.75 86.05 82.44	29.60 36.81 33.70	15.22 20.92 23.89	73.44 67.03	66.23 62.94	89.93 93.94	92.58 93.55
9 { Nitrogen free diet Paddy, parboiled " and parched ('kurmura')	0.042 0.888 0.930	4 4 4	3.31 98.80 93.18	18.73 30.26 26.62	15.71 22.65 20.20	88.53 85.38	76.50 79.00	86.46 90.68	92.63 94.68
10 { Nitrogen free diet Rice, polished Paddy, beaten ('pohe')	0.042 0.902 0.902	6 6 6	3.51 74.56 71.02	13.57 26.24 21.86	26.24 21.86 21.85	66.71 63.33	53.99 55.65	80.27 87.64	93.60 93.43
11 { Nitrogen free diet Paddy beaten and parched ('murmura')	0.042 0.914	6 6	3.35 79.85	17.69 32.19	16.29 18.86	73.91	59.44	80.44	96.91
Paddy parched ('ball')	0.002	6	78.00	10.55	18.78	7.01	50.76	82.20	96.91

## DISCUSSION

In Table II are given the biological values and the digestibility coefficients of unparched and parched foodstuffs which include five pulses, viz Bengal gram ('chana'), dried peas ('vatana'), black gram ('urd'), green gram ('mung') and horse gram ('kulthi'), and four cereals, viz 'juar', 'ragi', maize and rice. It can be seen from Table II, column 9, that the biological value of the proteins of Bengal gram on 10 per cent level of protein intake is less than that on 5 per cent level. Niyogi, Narayana and Desai (1931*a*, 1931*b*, 1932) have also observed that the biological value decreases with an increase in the level of the protein intake. All the experiments reported here have been done only at the 5 per cent level of protein intake.

Three groups of investigators, viz Niyogi and collaborators (*loc cit*) at Bombay, Swaminathan (1937) at Coonoor and Basu with co-workers (1936*a*, 1936*b*, 1937) at Dacca, have investigated biological values of the proteins of unparched cereals and pulses. The results reported by them agree very well with those found in the course of this investigation.

It would appear from Table II that the biological values show an improvement on parching. This effect is not uniform, however, on subjecting the results to statistical analysis it was found that the difference between the biological values of unparched and parched grains was significant only in the cases of green gram, horse gram, field pea among the pulses, and 'ragi' and 'juar' among the cereals. Of all the varieties of parched rice only the parboiled and parched variety ('kurmura') showed a significant increase over the raw milled rice. Unless some more information is obtained as to the reason for the observed increase of the biological value of proteins on parching the grain, it would be difficult to give an adequate explanation for the lack of uniformity in results mentioned above.

In cases where the B V has shown a significant increase on parching the digestibility coefficient did not necessarily show a parallel increase. On the other hand in some cases a slight decrease was observed. The point will be clear on referring to the case of green gram, the B V and the digestibility coefficient of which before and after parching are 50 and 70, and 86.9 and 85.20 respectively.

## SUMMARY

The biological values of the proteins of some unparched and parched cereals and pulses have been investigated by the balance sheet method using albino rats as experimental animals. All these experiments were performed on a 5 per cent level of protein intake.

It was found that parching (a) increased the biological value of the proteins of green gram, horse gram, field pea, 'juar', 'ragi' and parboiled rice and (b) in many cases improved the digestibility coefficient.

## ACKNOWLEDGMENT

The authors have great pleasure in thanking Dr Jivraj N Mehta, the Dean, Seth G S Medical College, Bombay, for his keen interest and encouragement

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## STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM

### Part V

#### THE ALLEGED INFLUENCE OF VITAMIN D ON THE ABSORPTION OF CALCIUM FROM THE INTESTINE EXPERIMENTS WITH ALBINO RATS

BY

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AND

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*(An Inquiry under the Indian Research Fund Association)*

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[Received for publication, August 5 1941]

RACHITIC infants are found to retain insufficient amounts of calcium and occasionally to be in negative calcium and phosphorus balance (Schabrad 1909, Birk and Orgler 1910, both quoted by Hess, 1930, Telfer 1922). The administration of sufficient amounts of cod-liver oil changes the negative balance to a positive one. This is achieved by a reduction of the faecal calcium subsequent to the administration of vitamin D (Telfer 1926). The net result is to make it appear that vitamin D increases the absorption of calcium and/or phosphorus from the intestine. Similar conclusions were reached by Watchorn (1930), Kern, Montgomery and Still (1931), Harris and Innes (1931) and others in studies on experimental rickets.

The majority of these studies were carried out, however, by the balance methods. The main difficulty in such types of investigations lies in the fact that it is not possible to evaluate separately the calcium absorbed and that excreted in the intestines since the channel of excretion and absorption happens to be the same in this case. Any variation, therefore, in the amount of calcium absorbed or excreted will affect the level of faecal calcium without indicating which of the two opposing processes was the determining factor. In the development of the technique of the isolated loop *in situ* by Verzár (1936) lay a

probable solution to the problem. Its use by Nicolaysen (1937) in studying the absorption of calcium in the normal and rachitic rats led him to support the current views about vitamin D action, for he observed that in rachitic rats the amount of calcium absorbed from the loops in a given time was less than that absorbed from the loops of the rats protected from rickets by vitamin D. Although Nicolaysen's work has yielded results in uniformity with the current views the evidence to the contrary cannot be lightly discarded. In 1926 Bergeim used an ingenious method for studying the absorption of calcium. He introduced an insoluble material, such as ferric oxide, together with calcium salts, and then determined the ratio of Fe to Ca at different levels of the small intestine. He observed that the deficiency of vitamin D did not adversely affect the absorption of calcium. Taylor and Weld (1932) have also provided experimental evidence to show that vitamin D does not influence the absorption of calcium from the intestine.

Further, a closer study of Nicolaysen's paper revealed certain flaws in his experimental methods which rendered it necessary to re-investigate this important question more thoroughly than it had been done hitherto. The technique chosen for this purpose was the same as was used by Nicolaysen but with certain modifications.

#### EXPERIMENTAL

*Technique*—The rats chosen for experiment were starved for 18 to 20 hours before they were used. The isolation of the loop was performed under ether narcosis. The accurate measurement of the length of the loop *in situ* was very difficult, hence as far as possible the entire length of the small intestine from pylorus to the cæcum was isolated by ligatures at both ends. Small glass-cannulas were passed in at these ends and the lumen washed carefully with 100 c c to 125 c c of warm physiological saline solution made in glass-distilled water till the washings were free from calcium. The fluid was gently pressed out between two fingers. The ends were then tied off and the cannulas removed. Two c c of a solution of calcium gluconate containing 12.3 mg of calcium were injected into the intestine near the pyloric end of the ligated loop by means of a syringe fitted with a very fine needle. The loop was then replaced in the abdomen, the walls closed, sutured and the narcosis discontinued. After three hours the rats were killed by chloroform, the loops removed and the length measured. A glass-cannula was passed from one end and the loop was washed with glass-distilled water till the washings were again free from calcium. This required about 150 c c to 175 c c of water. The washings were acidified, made to volume and the calcium estimated by volumetric permanganate method.

To ascertain the accuracy with which calcium gluconate solution could be delivered by the syringe several trials were carried out in which 2 c c of the solution were delivered into different beakers and the amount of calcium estimated. The results showed that the delivery from the syringe was quantitative and uniform. It was also essential to find out if all the calcium introduced into the intestine could be quantitatively recovered. For this purpose six rats were used in which the loops

were washed immediately after introducing the calcium salt. The estimation of calcium in the washings proved that it could be recovered almost quantitatively ( $98.63 \pm 2.22$  per cent)

The normal, hypervitaminotic, rachitic rats and those protected from rickets by vitamin D were employed for the investigation. In addition to these some rats bred from stock receiving vitamin D poor diet and continued on a similar diet after weaning were utilized.

(1) *Normal rats*—Seven adult rats reared on the stock diet were employed for the absorption experiments. In the Table are given the results obtained —

TABLE  
(12.3 mg Ca were introduced into the intestine)

	Age in days	Weight, g	Length of the loop, cm	CALCIUM ABSORBED IN THREE HOURS	
				mg	mg /cm
(A) Seven rats on stock diet					
Maximum	207	255	42.5	6.29	0.170
Minimum	200	130	30.5	3.01	0.071
Mean	203	208	37.2	4.86	0.133
$\sigma$				$\pm 1.20$	$\pm 0.030$
(A1) Four rats (mild hypervitaminosis D, 7,200 I U of vitamin D per rat per day)					
Maximum	254	207	39.0	4.23	0.120
Minimum	236	157	32.0	3.01	0.083
Mean	245	192	36.0	3.76	0.105
$\sigma$				$\pm 0.596$	$\pm 0.018$
(A2) Five rats (severe hypervitaminosis D 7,200 I U of vitamin D per rat per day)					
Maximum	234	182	45.0	4.96	0.140
Minimum	200	150	31.0	2.89	0.084
Mean	215	166	35.8	4.16	0.116
$\sigma$				$\pm 0.847$	$\pm 0.063$

Group (a) developed rickets within four to six weeks and were ready for experiments (that they had rickets was later confirmed by bone analysis). They were then utilized for the purpose of studying the absorption of calcium. The rats from the other control groups were also experimented upon at the corresponding intervals of time. The results of these experiments are given in Table B.

(4) *Rats kept on a diet deficient only in vitamin D* — Although rickets in rats can be produced by feeding the diets grossly deficient or unbalanced in calcium and/or phosphorus in the absence of vitamin D, this condition is seldom associated with clinical rickets. In the majority of such cases the cause of rickets is either a lack of vitamin D, the want of proper sunshine, or of both. Hence it was thought necessary to study the absorption of calcium under conditions in which a deficiency of vitamin D alone was the disturbing factor while the amount of calcium and phosphorus and the ratio Ca/P in the diet were maintained within normal limits. This was accomplished by breeding young ones from parents which were kept on a diet poor in vitamin D and having the following composition —

*Vitamin D poor diet*

	Grammes
Starch	50 0
Dried skimmed milk	20 0
Red palm oil	10 0
Sodium chloride	0 18
Marmite	1 0
Ascorbic acid	0 006

Thirteen young rats (four weeks old) were utilized for these experiments. They were divided into two groups: (a) one consisting of seven rats was given the vitamin D deficient diet only and (b) another of six rats which received 60 I. U. of vitamin D per day orally as a supplement (control). The rats in group (a) grew slowly and their weights became stationary or began to decrease at the end of nine weeks on the diet. They were then used to study the absorption of calcium. The rats of group (b) (control) were also experimented upon at corresponding intervals. In Table B1 are given the results of these experiments.

The experimental data on the absorption of calcium from the isolated loops have been treated statistically according to Bradford Hill's (1939) method for small numbers. As the absorption from the intestine is likely to be influenced also by the area of the intestinal surface which in its turn would be proportional to the length of the loop, it was hoped that the comparison on the basis of calcium absorbed per unit length of the loop might probably yield more correct information on the point. Hence a comparison has been made on that basis also.

### DISCUSSION

It has been stated earlier that certain flaws were noticed in the technique used by Nicolaysen (*loc cit*) to study the absorption of calcium from the intestines of

rachitic and normal rats. He found it impossible to rinse all the remaining calcium out of the lumen at the end of the experiment. The loops from which calcium had been absorbed and which were rinsed and emptied, were found by him to contain about 0.6 mg Ca per g of loop as against 0.1 mg per g of loop in which no calcium had been introduced. Hence, in order to recover this amount he ashed the entire loops with nitric and phosphoric acids and estimated the total calcium from the ash and the washings as the unabsorbed calcium. Although this procedure ensured the recovery of all the amount in the lumen, it probably added a fresh source of error in the evaluation of his results. During the process of absorption calcium is taken up by the villus epithelium and then transferred by the blood capillaries and the lymphatics to the general circulation (Chitre and Patwardhan, 1940). As the process must be continuous during absorption, at any given moment some calcium has passed into the lymph and blood outside the intestinal walls and some would still be in the lymphatics and capillaries of the intestines waiting its turn to be transferred. Both these quantities together should represent the absorbed calcium. If, however, the intestine is ashed as was done by Nicolaysen and the amount of calcium found in the ash added on to the unabsorbed calcium recovered by washing from the lumen, it will give an entirely wrong picture, because by ashing the intestine the amount of calcium which had been absorbed but not yet left the intestinal walls will also be counted as unabsorbed calcium, a phase it does not actually represent. It may be pointed out that in the present investigation it was possible to recover immediately after introduction the total amount of calcium introduced, the recovery being  $98.63 \pm 2.22$  per cent. It is clear therefore that the extra 0.6 mg Ca per g of loop recovered by ashing should not have been included in the value for unabsorbed calcium. Further, the number of rats used by Nicolaysen was small and the results were not subjected to statistical analysis.

Another possible source of error in the evaluation of results obtained by the loop technique lies in the probability of some excretion of calcium in the tied loop during the period of the experiment. This aspect has been investigated by leaving the washed and tied loops *in situ* without introduction of the calcium salt. At the end of three hours the loops were washed as described and calcium estimated in the washings. Several such experiments indicated that only negligible amounts of calcium were excreted in the loop in three hours, the average being 0.2 mg for the entire loop.

The results presented in the Table would seem to indicate that there are no 'significant' differences between the total amounts of calcium absorbed in the three groups, viz 'rickets' ( $5.63 \pm 1.56$ ), 'rickets protected by dosage with vitamin D' ( $6.14 \pm 2.27$ ) and the 'stock group' ( $4.21 \pm 1.24$ ). When the comparisons were made between the groups of rats on the 'rachitogenic diet' and those on the stock diet on the basis of calcium absorbed per cm of the loop there was found a significant difference, but in this case the value was higher in the rachitic group than in the 'stock group'. The mean value of calcium absorbed per cm. of loop was also higher in rats receiving the 'rachitogenic' diet supplemented with vitamin D as compared with those on stock diet, although this difference was not significant. No suitable explanation can be advanced for this



peculiar behaviour of rats on rachitogenic diet with or without vitamin D. The experiments on rats kept on a diet deficient in vitamin D but otherwise adequate also failed to show 'significant' difference between the rates of absorption of calcium in the 'avitaminotic' and the 'control' groups.

Similarly, the attempts to demonstrate the effects of excessive dosage of vitamin D for short or long periods on the absorption of calcium from the intestine of rats have failed to show any significant difference between the normal and hypervitaminotic rats.

In view of the above findings it is considered necessary to find a satisfactory explanation for the commonly observed increase in the faecal calcium in the state of vitamin D deficiency. It is suggested that although vitamin D may not exert a direct influence on the absorption of calcium, it is possible that it may do so in an indirect manner by altering the condition or conditions in the intestinal tract, which in a normal animal are not unfavourable for the absorption of calcium. It must be borne in mind that the experiments described above were carried out with the calcium salts alone in absence of any extraneous disturbing factors. It is known, however, that hydrogen ion concentration of the contents of the small intestine, the presence of varying quantities of fat, the ratio of Ca to P, etc., are some of the factors which determine the extent of the absorption of calcium from the intestine. Considerable work on these problems has been published by various authors. In the main it has been inconclusive. It will be reviewed in a later communication dealing with the examination of the factors believed to influence the absorption of calcium from the intestine.

### SUMMARY

The absorption of calcium salts from the intestines of albino rats by the isolated loop technique has been studied. Observations were made on normal and rachitic rats as well as on the rats with induced hypervitaminosis D and the results statistically treated. No evidence of any direct influence of vitamin D on the absorption of calcium could be obtained.

### ACKNOWLEDGMENTS

The authors have great pleasure in thanking Professor S. P. Niyogi and Dr. Jivraj N. Mehta, the Dean, for their interest and encouragement.

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## OBSERVATIONS ON THE DIET AND NUTRITIONAL STATE OF AN ABORIGINAL (HOS) TRIBE

BY

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[Received for publication, September 18, 1941]

### INTRODUCTION

In two previous communications from this laboratory (Mitra, 1940a, 1941) dealing with the dietary of people in industrial areas of Bihar, it has been shown that rise in income is usually, though not always, followed by greater consumption of all the protective foods essential for the maintenance of optimum health. In the present study an attempt has been made to find out how groups of aboriginal families behaved when they could afford to spend more money on food.

The aboriginal tribe in question were the 'Hos' who are said to have migrated to the district of Singhbhum several centuries ago. Their numerical strength was given as 301,158 persons (or 32.4 per cent of the total population of the district) at the 1931 census. The district of Singhbhum is situated at the south-eastern corner of the Chota Nagpur plateau in the province of Bihar. Rice is the principal crop grown in the district. A small quantity of millets, pulses and oil seeds is also grown.

### FAMILIES SURVEYED

An investigation into the food intake of 250 Ho families consisting of 1,272 persons was carried out during the period November 1940 to April 1941 on the lines suggested by Aykroyd and Krishnan (1937). Each family was under observation for ten days only. One hundred and fifty families were sampled from villages in the neighbourhood of Chakardharpur, a district headquarters of the B. N. Ry., and 100 families from the villages situated close to Jagannathpur, a big village possessing a charitable dispensary and an English High School. In selecting the sites for the survey, Chakardharpur and Jagannathpur were chosen as in both these areas the Hos were earning their livelihood by following agricultural pursuits in comparatively fertile level tracts and were uninfluenced by industrial development. The earners in these families consisted mainly of tenant cultivators and agricultural labourers.

The total income in each of the families was assessed on the lines detailed in a previous communication (Mitra, 1940b) and the average income per consumption

unit calculated on the basis of the International scale of family coefficients suggested by the League of Nations (1932) This index has been used for classifying the families in different income level groups as the total income of the family fails to convey any idea as to the *per capita* spending power of each family unit The families were classified into four groups (a) group I, i.e. those families with an annual income up to Rs 30 per consumption unit per annum, (b) group II, i.e. those up to Rs 60, (c) group III, i.e. those up to Rs 90, and (d) group IV, consisting of families with an income of over Rs 90 The average annual income per consumption unit (c u) for the four groups was worked out For the sake of brevity group numbers will be given when referring to any one of the income groups later in the text

TABLE I

*Details of families in the different income groups*

Group numbers	Number of families in each group	Limits of annual income per c u	Average number of consumers per family	Average number of 'man value' consumption units per family	Average annual income per c u. in rupees.*
I	91	Up to Rs 30	5.6	4.2	21.5
II	121	Up to Rs 60	5.0	4.5	43.1
III	26	Up to Rs 90	4.0	2.1	72.4
IV	12	Over Rs 90	4.4	3.4	159.9

\* One rupee equals 1s 6d (approx)

## CLASSES OF FOODSTUFFS CONSUMED

The average daily intake of the different classes of food per c u in ounces, in each of the family groups, is shown in Table II —

TABLE II

*Daily consumption per c u in oz*

Family groups	Cereals	Pulses	Leafy vegetables	Non leafy vegetables	Oils and fats	Flesh foods	Condiments
I	17.8	0.6	0.5	0.4	*Neg	Neg	0.1
II	19.5	0.8	0.9	0.5	Neg	Neg	Neg
III	21.2	0.8	1.1	0.5	Neg	Neg	Neg
IV	23.0	0.9	0.9	0.7	Neg	Neg	Neg

\* Neg = negligible or less than 0.1 oz

Home-pounded parboiled rice was the only cereal grain consumed. The cooking water or *marh* was not thrown away but consumed along with rice. One hundred and twenty families (or 48 per cent) did not consume any pulses. Every Ho family maintains a kitchen garden. Lufu or dried tender shoots and leaves were very popular. The use of the potato was almost unknown, but some amounts of colocasia and other stem or root tubers were eaten. Tomato was found to be a popular edible but the production of this vegetable as also of others was very small. Two hundred families (or 88 per cent) were found to use no oils or fats for culinary purposes.

Though the Hos entertain no prejudice against any of the meat foods, only six families were found to consume fowls and two families duck's eggs from their own poultry. Only one family consumed small fish.

The amount of wild fruits which is consumed during the different seasons could not be estimated and has not been taken into consideration.

The average intake of calories per consumption unit *per diem* in family groups I to IV were found to be 1,907, 2,096, 2,263 and 2,454 respectively. While the families under investigation follow agricultural pursuits involving very little labour as they depend almost entirely on rainfall or diversion of flood water from rivers and the number of working days barely exceeds 150 in the year, yet people consuming less than 2,500 calories per c u *per diem* must be regarded as subsisting on a quantitatively insufficient diet. According to this standard about 64 per cent of the families in group I, 46 per cent in group II, 35 per cent in group III and only 3 per cent in group IV were found to fall below this level of calorie intake. This finding, along with the increase in average intake for each group justifies the conclusion that with increase in income the calorie intake tends to become more and more satisfactory.

#### ESTIMATION OF NUTRITIVE PRINCIPLES

The amount of the various nutrient principles consumed per c u *per diem* are shown in Table III —

TABLE III

*Average daily intake per c u in the different income groups*

Family groups	Protein, g	Fat, g	Carbohydrate, g	Calcium, g	Phosphorus, g	Vitamin A, I U	Vitamin B <sub>1</sub> , I U	Vitamin C, mg
I	48.7	2.4	411.0	0.162	1.517	1.145	532	15.4
II	53.9	3.3	447.9	0.226	1.616	2.113	577	23.3
III	58.7	3.0	486.4	0.250	1.795	2.152	630	26.3
IV	63.3	3.8	526.8	0.194	1.912	1.816	718	31.4

The intake of calories, protein and fat was found to maintain a positive correlation with the income

With the exception of the carbohydrate, phosphorus and vitamin B<sub>1</sub>, the other elements in the diet seem to be very inadequate. The phosphorus figures on their face value seem to be satisfactory but, with the exception of an extremely negligible percentage, the whole of this element was derived from vegetable sources and therefore comparatively unavailable. The figures for vitamin C may appear adequate but the calculations have been based on raw food and the thermolability of this particular factor is well known. The quota of fat in the diet was lower than the lowest figure of consumption recorded so far, i.e. that recorded in the case of another aboriginal tribe (Mitra, 1940b), though there is a slight suggestion of increasing consumption with rise in income.

### DISCUSSION

It is a truism that poverty is primarily responsible for shortage of calories, or in other words, quantitative deficiency in the diet. In the families classed under groups I and II the average daily income per c u comes to 113 pies\* and 19 annas respectively, and hardly any improvement can be expected unless the earning capacity be increased. In the case of families classed under groups III and IV the average incomes per c u *per diem* were found to be 32 and 70 annas respectively and it was expected that a more liberal consumption of calories would be observed in these two groups, on the basis of observation on the calorie consumption in family groups of similar income level in the same district (groups I and III in Jamshedpur, Mitra, 1940a), or in the contiguous district of Manbhum (groups II and III in Jharia, Mitra, 1941). In each of the groups of families in these industrial areas, the average daily consumption per c u was in the neighbourhood of 3,000 calories. The fact that people engaged in industrial work required a greater calorie consumption does not fully explain the discrepancy. It must, however, be noted that in the case of another aboriginal tribe, the Santhals (Mitra, 1940b), though these were sampled from slightly lower income levels as compared to groups III and IV in the present study, the average intake of calories was found to vary between 1,378 and 2,275 in all the (seven) groups. Whether, in the case of aboriginals occupied in agricultural pursuits, the appeasement of hunger is always the surest guide to quantitative requirements can be proved only by further study.

The qualitative deficiency in the dietary is usually caused by (a) ignorance, (b) respect for traditional dietary habit and (c) scarcity of protective foods in addition to poverty.

In order to decide how far each of these facts was responsible for the deficiencies noticed particularly in groups III and IV, it must be recorded that milk and milk products are not available in the area for love or money. The Hos themselves use cows (along with bullocks) for ploughing fields in preference to their use as milch

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\* One rupee is equivalent to sixteen annas or 192 pies

cattle Owing to lack of demand sufficient vegetables were not available in the weekly or bi-weekly village markets Except for chicken, flesh foods are rarely sold, and chickens are eagerly purchased by non-aboriginals for what is considered to be a good bargain The produce from the kitchen garden is poor for various reasons Under the circumstances the Hos in the comparatively higher economic scales cannot obtain a better diet if they want to unless the environment is changed

The most widely used items of food, e.g. pulses and vegetable oil, are available in plenty in all the *hatias* (markets) and nothing except faulty food habit prevents the Hos from including these things in the diet The only methods of cooking known to them are boiling all edibles, or, at times, baking Elementary methods of cooking curry or gruel from pulses and other preparations were found to be unknown to these people Consequently they used no condiments such as turmeric, coriander, red chillies, etc Increase in income made no difference as to the degree of culinary art practised or as to the devising of a more varied menu

TABLE IV

*Frequency distribution of families consuming not more than two items of foodstuff*

Family groups	FAMILIES CONSUMING THE FOLLOWING —					TOTAL.
	Rice	Rice and tamarind.	Rice and one kind of pulse	Rice and one kind of non leafy vegetable	Rice and one kind of leafy vegetable	
I	6	5	14	6	16	47 (51·7 per cent)
II	6	1	16	8	20	51 (42·1 per cent)
III	1	<i>Nil</i>	3	<i>Nil</i>	2	6 (23·1 per cent)
IV	<i>Nil</i>	<i>Nil</i>	1	1	1	3 (25·0 per cent)
All groups	13	6	34	15	29	107 (42·8 per cent)

Table IV is an interesting study and proves that about one-fourth of the families in groups III and IV were found to consume only one kind of food in addition to rice Thirteen families were found to subsist on rice alone without any supplement Ignorance and adherence to traditional dietary habit can only be held responsible for this state of affairs

It is justifiable to conclude that increase in income was not followed by any qualitative improvement in the diet consumed by the Ho families under investigation

Dalton (1872), who is said to be an authority on the Hos, observed about 70 years ago that 'the Hos of Singhbhum' were physically a much finer people



than the Bhumijs, the Santhals, or any other Kolerians. According to him the males averaged 65 to 66 inches in height and the women 62 inches. During the present investigation an attempt was made to measure the height and weight of the adults. The mean height in inches on the examination of 503 males (the females refused to co-operate) was found to be  $62.54 \pm 0.11$  S.E. with a standard deviation of  $2.00 \pm 0.08$  S.E. The mean weight in pounds was found to be  $99.52 \pm 0.57$  S.E. with a standard deviation of  $10.4 \pm 0.41$  S.E. A comparison of these figures with the respective figures for Santhal males compiled by the author (Mitra, 1940b) shows that there is no significant difference between the average stature and weight of the two tribes.

Dalton, who was a Colonel in the militia raised from aboriginal tribes some time before he was deputed to study ethnology in Chota Nagpur, does not say how he obtained these figures. One is thus left wondering whether the physique of the Hos has deteriorated because of the consumption of such a poor diet or whether Dalton's figures were not representative of the whole population.

In order to study the effect of such a poor diet, 2,403 Ho children were rated clinically and examined for deficiency diseases on the lines detailed in a previous communication (Mitra, 1940a). It was found that 32.9 per cent of the boys and 28.6 per cent of girls were rated as poorly nourished and this percentage was found to be higher than the highest figure so far recorded (in the case of Paharia children, Mitra, 1940b) in the province. As regards apparent signs of dietetic deficiency 12.7 per cent of the boys and 8.1 per cent of the girls were found to be suffering from either phrynoderma, xerophthalmia or angular stomatitis.

The teeth of all the children were examined by dental probe and mirror for the presence of caries and malocclusion of teeth. 13.5 per cent of the boys and 15.4 per cent of the girls were found to be suffering from dental caries. The percentage incidence of malocclusion of teeth in the boys and girls were found to be 20.9 and 24.8 respectively.

*Knudsen-Schlotz index*—The dorsal median furrow of all the children were examined according to the methods detailed by Bigwood (1937).

TABLE V.

*Assessment of nutrition by Knudsen-Schlotz index*

Sex	Number examined	SATISFACTORY		UNSATISFACTORY	
		Actual	Percentage	Actual	Percentage
Boys	1,668	1,200	71.9	468	29.1
Girls	735	560	76.2	175	23.8
Both	2,403	1,760	73.2	643	26.8

The percentage of children diagnosed to be in an unsatisfactory state of nutrition by this method is slightly lower than that of children diagnosed as poorly nourished by naked-eye rating

### SUMMARY

1 A dietary survey of 250 Ho (aboriginal) families consisting of 1,273 persons was carried out in Singhbhum district from November to April. The families were classified into four income groups and the average annual income per consumption unit in these groups was found to be Rs 21.5, Rs 43.1, Rs 72.4 and Rs 159.9 respectively.

2 Sixty-four per cent of the families in group I, 46 per cent in group II, 35 per cent in group III and only 3 per cent in group IV were found to be consuming diets quantitatively deficient.

3 The intake of calories, protein and fats was found to maintain a positive correlation with the rise in income level. Though the people entertain no prejudice against flesh foods, very little of these was actually consumed. Eighty-eight per cent of the families used no oils or fats in cooking food.

4 The consumption of milk and milk products was unknown. Only a negligible quantity of vegetables was consumed. The quality of diet in most of the families was inadequate. Rise in income made no difference.

5 A little more than 2,400 children were also examined to discover the incidence of various deficiency diseases. By naked-eye rating about 32 per cent of the children were found to be poorly nourished, whereas by the Knudsen-Schiøtz index about 27 per cent were found to be in a state of unsatisfactory nutrition.

6 The survey proves that increase in income is not always associated with qualitative improvement in the diet. Ignorance and regard for traditional dietary habits may prevent such an improvement taking place.

### ACKNOWLEDGMENTS

The author is obliged to Dr N. P. Verma for his help in carrying out nutrition survey, to Dr W. R. Aykroyd for kindly revising the manuscript and to his chief, Rai Bahadur Dr B. P. Mozoomdar, for his advice and encouragement.

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## SOME OBSERVATIONS ON THE ACTION OF CAMPHOR, CORAMINE AND CARDIAZOL ON THE CIRCULATION AND RESPIRATION

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[Received for publication, July 25, 1941]

CAMPHOR has been used for a long time as a temporary cardiac stimulant. Opinions are divided as to its usefulness, because the effects, if they occur, are inconstant and unreliable. The literature regarding its cardiac and respiratory effects is voluminous, but at the same time very contradictory, and no purpose is served by quoting from it here. That camphor in oil given by injection can produce a temporary stimulation of the pulse and respiration cannot be controverted, but this, as pharmacological experiments show, is in the ordinary doses only a reflex phenomenon and, therefore, cannot be made use of for routine stimulation of either the heart or respiration.

In order to get over the insolubility of camphor, many soluble preparations have been devised. Cardiazol (pentamethylene tetrazol) is a synthetic product with similar but more powerful action on the heart and respiration, both reflex as well as direct. Hexetone, Cardatone, a sodium derivative, and Solucamphre (Delalande), which is a d-campho sulphonate of diethylene diamine, are among the more recent derivatives introduced into therapeutics.

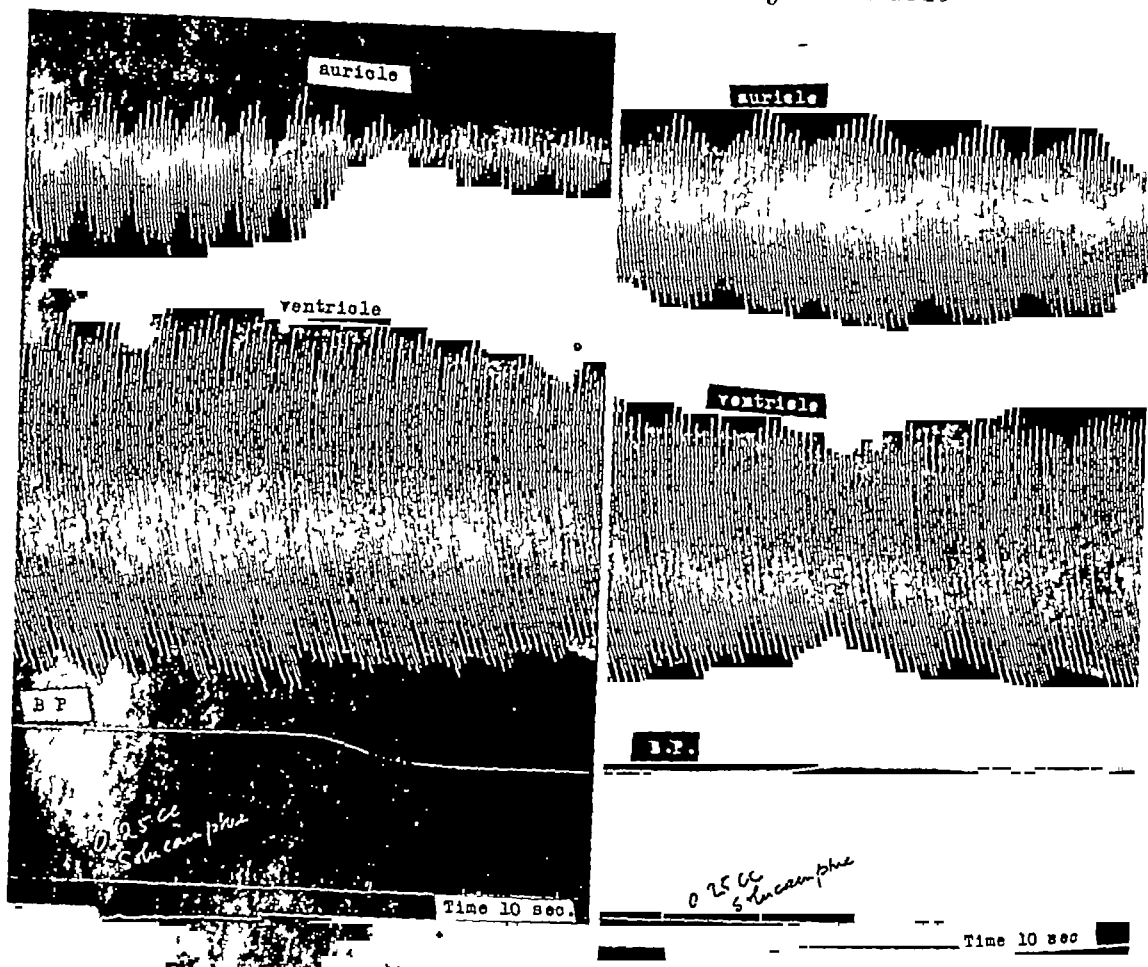
**SOLUCAMPHRE**—Some experiments on the pharmacological action of Solucamphre (Delalande) on the heart and respiration may first be mentioned.

*On the heart*—Myocardiograph records of the cat's heart under anaesthesia show a temporary inhibition and depression of the heart muscle, much more marked

on the auricle This effect is completely abolished after atropinization (Graph 1) We know that the vagus effect on the heart is mostly seen on the auricle, and hence we see the vagus action of Solucamphre as a much greater depression of the auricle than the ventricle On the blood pressure the effect is variable Ordinarily, after an intravenous injection into cats and dogs a fall of blood pressure is noticed In

GRAPH 1

Cat 25 kg Chlorotone intraperitoneally 25-9-1940



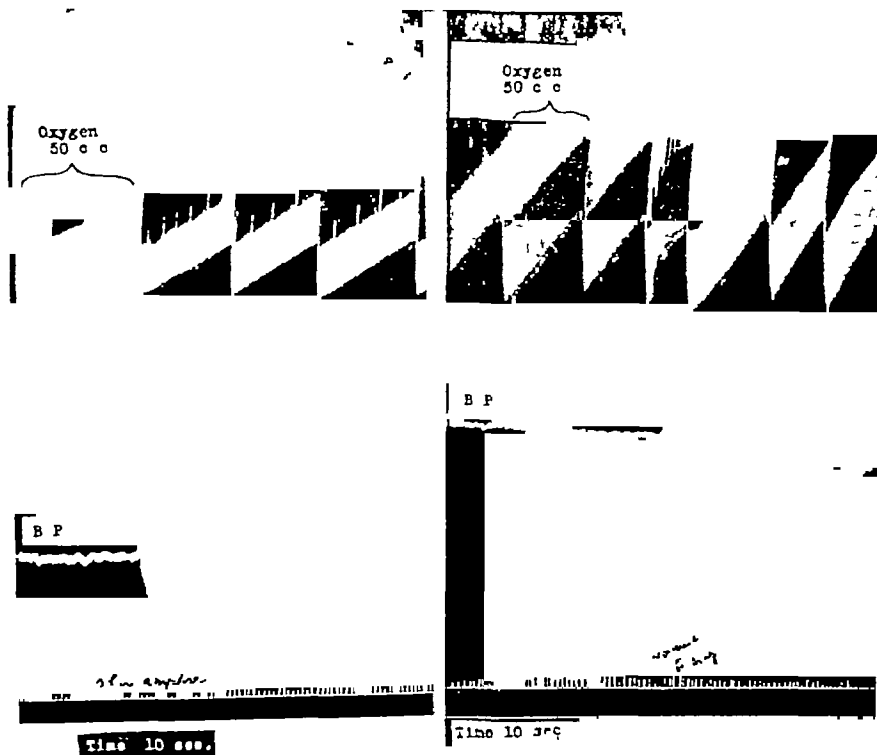
(a) 0.25 cc Solucamphre

(b) 0.25 cc Solucamphre after 0.5 mg Atropine

cats, the fall is either completely or to a great extent prevented after atropinization (Graph 1) An explanation for the fall of blood pressure which, though smaller than before, is still noticed after atropinization is that it is due to peripheral vasodilatation caused by camphor and its derivatives, also seen in the case of Solucamphre (Delalande)

Solucamphre (Delalande) is also supplied by the manufacturers in combination with strychnine, caffeine or ephedrine with the idea that such combinations may produce a potentiation of the combined action. We noticed that while there was an additive action on the circulation, as well as respiration, there was no potentiation. The cardiac action of caffeine and the respiratory stimulation of strychnine could both be noticed without any special reinforcement due to the action of Solucamphre as claimed by the manufacturers.

GRAPH 2

*Oxygen consumption*

- (a) Cat 21 kg Chloretone intraperitoneally 20.9.1940  
1 c.c Solucamphre
- (b) Cat 2.99 kg Urethane 30.6.1941  
0.25 c.c Coramine (Dr Dey)

*On the respiration*—Solucamphre certainly produces a stimulation of the respiration as seen from a quickening and increased amplitude of the respiratory movements as well as increased oxygen consumption (Graph 2). This effect persists after atropinization showing an action on the medullary centres. Here there seems to be a true stimulation of the centre concerned.

The record of the consumption of oxygen (Graph 2) was taken by the floating pan spirometer as described by Jackson (1939)

Solucamphre is therefore a stimulant of the respiratory centre quickening the rate and amplitude of breathing with a correspondingly increased consumption of oxygen. Therefore it would be of clinical value in cases where such stimulation is indicated. Solucamphre cannot, however, be classed as a cardiac or circulatory stimulant though it can act as a general diffusible reflex stimulant, like injection of ordinary camphor.

CORAMINE or Nikethamide (Pyridine B-carbonic acid diethylamide) is another popular stimulant of the respiratory centre. We experimented with two samples of this preparation, one prepared in the chemical laboratory of the Presidency College, Madras, kindly sent to us by Professor B. B. Dey, and another marketed under the name of 'Cardiamid' (Cipla), both in 25 per cent solutions.

We found a very marked and immediate increase in the oxygen consumption and in the amplitude of the respiratory movements (Graph 2). Coramine is therefore a real stimulant of the respiration.

On the heart, Coramine produces no demonstrable stimulation. On the other hand, we find in experiments on intact and isolated hearts that there is a definite depression of rate and amplitude instead of stimulation, and there is always a corresponding fall of blood pressure which is rather temporary.

CARDIAZOL or Leptazol. Experiments in this laboratory with Cardiazol (Knoll) and Corasol (Cipla) confirm our previous findings (David and Vareed, 1929) that this drug is a real stimulant of the respiratory centre and to a smaller extent of the heart. Even large doses do not depress the heart, but unfortunately tend to produce convulsions.

We thank the manufacturers, Michel Delalande of Paris and 'Cipla' Laboratories, Bombay, for supplying us with a certain amount of these preparations for study through their local representatives.

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A PRELIMINARY NOTE ON THE PHARMACOLOGICAL  
ACTION OF THE GLUCOSIDES FROM  
*PARIS POLYPHYLLA* SM

BY

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[Received for publication, September 22, 1941 ]

INTRODUCTION

THIS liliaceous plant grows abundantly in the temperate regions of the Himalayas from Simla to Bhutan. The plant is not mentioned in the Watt's Dictionary of the Economic Products of India and no information is available regarding its medicinal properties and uses.

The roots of this plant secured from Nepal have lately been investigated by the Department of Chemistry of this Institution and two glucosides allied to paridin and paristypnin, found in *Paris quadrifolia* Linn, have been isolated. The details about the isolation of these two glucosides have already appeared in a paper (Dutt, Chatterjee, Ghosh and Chopra, 1938) and a paper dealing with the properties and composition of the two glucosides will soon appear in a separate communication.

Since the composition and properties of the glucosides isolated from this plant do not agree in many details with those of paridin and paristypnin, the present authors have suggested the names  $\alpha$ -paridin and  $\alpha$ -paristypnin for the glucosides of *P polyphylla*.



*Properties of the glucosides* — *α*-paridin crystallizes from alcohol in glistening laminae m p 244°C to 246°C It produces a slight tingling sensation on the tongue It is very slightly soluble in cold water but more so in hot water It is sparingly soluble in cold alcohol but fairly soluble in hot alcohol *α*-paristypphin forms a cream coloured amorphous powder m p 147°C to 149°C It is freely soluble in water and alcohol and produces a tingling sensation on the tongue

### EXPERIMENTAL RESULTS

The action of the two glucosides was studied on the cardio-vascular and pulmonary systems, the spleen, kidneys, intestine and limb volumes and on the isolated intestine of guinea-pig

I *Action of α-paristypphin* — 1 *Pressor effect* — In normal chloralosed cats, *α*-paristypphin produced an initial fall of blood pressure amounting to 10 mm Hg, 14 mm Hg and 28 mm Hg in 2.5 mg, 5 mg and 10 mg doses respectively This was soon followed by a secondary rise not exceeding, however, 16 mm Hg above normal Paralysis of the vagal or sympathetic nerve-endings by atropine or ergotoxine did not modify the above effects

2 *Cardiac action* — Myocardiograph experiments showed that in doses stated above the amplitude of both auricular and ventricular contraction considerably decreased and the heart temporarily dilated The rhythm remained unaffected

3 *Intratracheal pressure* — *α*-paristypphin was found to be a marked respiratory depressant and reduced considerably the amplitude of respiratory movements, but did not change the rate

4 *Spleen volume* — This uniformly increased from vaso-dilatation in the organ

5 *Kidney volume* — Unlike splenic dilatation oncography demonstrated evidences of vaso-constriction and the volume of the organ was diminished

6 *Intestinal volume* — Initial vaso-constriction was soon followed by a secondary vaso-dilatation in the organ

7 *Limb volume* — *α*-paristypphin produced a definite vaso-dilatation in the periphery

8 *Isolated guinea-pig intestine* — In a concentration of  $10^{-5}$  *α*-paristypphin accelerated peristalsis but the tonus remained unchanged In higher concentrations, such as  $0.5 \times 10^{-5}$  and  $0.25 \times 10^{-5}$ , tonic contractions were obtained, the intensity of which was roughly proportionate to the doses utilized The contraction was slow but progressive and ceased on washing This stimulating action of *α*-paristypphin was not inhibited by atropine

II *Action of α-paridin* — This was studied on the very same organs as in cases of *α*-paristypphin *α*-paridin was found to possess some pressor effect but much less marked than in case of *α*-paristypphin On the isolated guinea-pig intestine, it stimulated the tonus without accelerating the peristalsis Pharmacologically, this glucoside could be considered to be much less active than *α*-paristypphin

### DISCUSSION

Analysis of various effects produced by glucosides occurring in *Paris polyphylla* shows that  $\alpha$ -paristypnin has got depressant actions on the carotid pressure, myocardium and respiratory movements. It produces vaso-constriction in the kidney but vaso-dilatation in the spleen and limbs. The fall in carotid pressure could possibly be explained by this latter phenomenon. Neither atropine nor ergotoxine modifies these actions. It is thus evident that the peripheral autonomic nervous system does not play any part in the mechanism of the cardio-vascular action of  $\alpha$ -paristypnin. This is also true for the isolated guinea-pig intestine where the stimulating action of  $\alpha$ -paristypnin is not modified by the effect of atropinization.

### CONCLUSIONS

1. Of the two glucosides of *P. polyphylla*  $\alpha$ -paristypnin is pharmacologically more potent than  $\alpha$ -paridin.

2. The different actions of  $\alpha$ -paristypnin do not appear to depend on the peripheral autonomous nervous mechanism. It acts, to all probabilities, directly on non-striated muscle fibres.

### ACKNOWLEDGMENTS

Our thanks are due to Dr S Ghosh and his staff for the isolation of the glucosides and to Mr R L Badhwar for procuring the plant from the Nepal States.

### REFERENCE

- DUTT, A. T., CHATTERJEE, N. R., GHOSH, S., *Arch. Pharm.*, **276**, p. 343  
and CHOPRA, R. N. (1938)

*Chemical nature and toxicity of cerberin* —The seeds contain a glucoside named cerberin isolated first by deVry which is sparingly soluble in water. The saturated solution has an approximate concentration of 1 in 5,000 only. This solution is non-irritating to the skin, conjunctiva and subcutaneous tissues but produces vomiting, diarrhoea and even syncope in animals if given by subcutaneous injection. The lethal dose was found to be 1.8 mg for the dog, 3.1 mg for the cat and 50 mg/kg for the rabbit.

### EXPERIMENTAL

A preliminary study of the cardio-vascular and muscular effects of the drug formed the main object of our investigation.

#### I Cardiac action

This was studied both on the isolated mammalian heart by the perfusion method of Langendorff as also by myocardiography of the heart *in situ*.

1 *Perfusion of the kitten heart* —The action of cerberin varies according to the magnitude of the dose utilized. In a concentration of  $10^{-6}$  it produces a marked cardiotonic effect on the amplitude of the ventricular contraction. This is increased to the extent of 30 to 40 per cent over the initial excursions (inotropism positive). The rhythm remains unchanged. At higher concentrations, such as  $10^{-5}$ , the initial stimulation is soon followed by a gradual depression (inotropism negative). Finally, the rhythm is altered and bradycardia sets in (chronotropism negative).

2 *Perfusion of the rabbit heart* —The effective dose for rabbit heart was found to be much higher. Stimulation was obtained with cerberin in the concentration of 1 in 40,000 and the amplitude nearly doubled at this dose (inotropism positive). In 1 in 20,000 doses the amplitude remained unchanged. Bradycardia was produced (chronotropism negative). Finally, disturbance of rhythm occurred and bathmotropism became negative.

3 *Myocardiography* —On the normal cat's heart in doses of cerberin, in doses varying from 0.5 cc to 1.5 cc, stimulation of the auricular and ventricular contractions after a preliminary stage synchronous with the fall of the carotid pressure. Depression of the rhythm occurred in the next stage and fibrillation was produced. The above effect was inhibited by atropine.

#### Vascular action

Effects of cerberin on spleen and kidney volume.

1 *Action on the cat* —On normal animals, total dose 2.5 kg and 3 kg approximately.

pressure of normal blood studied.

This was studied in cats of 2.5 cc to 1.5 cc weight. A fall in blood pressure was observed.

from 10 mm to 15 mm Hg. This is immediately followed by a rise of from 20 mm to 25 mm Hg, the curve resembling that met with in case of saponins. Atropinization inhibits the first action.

2 *Action on organ volumes*—(a) *Plethysmography* The spleen volume is increased by cerberin in the above doses and is synchronous with the rise of the carotid pressure. (b) *Oncography* The volume of the kidney remains unchanged in above doses but the normal rhythmic movements, consisting of alternate contraction and dilatation, are increased in rate and amplitude.

### III Action on plain muscles

The cardio-vascular action of cerberin and the effect of atropinization suggested that the action of cerberin on the plain muscles of intestine should also be investigated. This was done on the isolated gut of the cat and guinea-pigs.

1 *Isolated guinea-pig intestine*—Cerberin, in concentrations of  $10^{-6}$  to  $5 \times 10^{-5}$ , stimulates the intestinal musculature. It increases both the tonus and the peristaltic movements. These actions are perfectly antagonized by atropine. Pilocarpine, on the other hand, increases the action of cerberin. In fact, intestinal strips previously treated with pilocarpine  $10^{-6}$  and washed show greater sensitiveness to the effect of cerberin. This is evidenced by decreased latent period and greater intensity of contraction and peristalsis. It would appear that pilocarpine acts synergistically with cerberin and potentiates its action.

2 *Isolated kitten intestine*—Cerberin also stimulates this organ. In  $5 \times 10^{-5}$  concentration the tonus is slightly increased and the amplitude of peristaltic movements is stimulated. In higher concentrations such as  $2 \times 10^{-5}$  a summative effect is produced.

### SUMMARY AND DISCUSSION

From this brief survey of the effects of cerberin it is evident that pharmacologically it is a potent drug. Its main effect is cardio-vascular. It acts both on the rhythm and the amplitude producing a positive inotropism in moderate doses and a negative ino, chronodromo and bathmotropism in toxic doses. These characteristics remind us of its partial resemblance with digitalis action and it would be interesting to detail these studies both clinically and experimentally and ascertain if it could claim any place as a digitalis substitute.

On the carotid pressure the action of cerberin is biphasic. It first depresses and then stimulates the pressure. It would appear that the initial fall is purely vagal as this disappears on atropinization. The secondary rise is more likely to be due to the direct muscular effect of the drug.

On the plain muscle of the intestine, cerberin acts as a definite stimulant both with regard to its tone and peristaltic movements. This action is somewhat similar to that of pilocarpine. Atropinization removes this vagal action, pilocarpine potentiates it. The above-mentioned stimulating action of cerberin on the intestinal musculature could possibly justify its empirical use as a cathartic.

Schmitz and Glover (1927) observed that the rate of glycolysis in normal blood varies between 15 mg and 25 mg per 100 c c per hour at room temperature and that the rate of glycolysis is not dependent on the initial blood-sugar level. They also made a suggestion that it was only the myelogenous leucocytes which are actively glycolytic.

Falcon-Lesses (1927) studied the glycolysis in heparinized normal blood, incubated at 37°C, of 9 healthy persons *in vitro* and observed that glycolysis is complete in 6 hours in samples of blood with an initial glucose content below 100 mg per cent.

Reid and Narayan (1931) studied the glycolysis of blood incubated at 37°C. They found that starvation decreases and food intake increases the rate of glycolysis in blood. They further observed that glycolytic factors are present in both erythrocytes and serum.

De and Bhattacharyya (1938) found that glycolysis takes place at the rate of 3.8 mg to 6.5 mg per hour per 100 c c of blood in normal blood and 4.0 mg to 16.6 mg per hour per 100 c c in diabetic blood. They further observed that complete glycolysis does not take place even after 24 hours' incubation at 37°C.

From the short review of the literature given above it thus appears that there is a fairly wide diversity of opinion among different workers as to the rate of disappearance of sugar from the blood *in vitro*, when the same is left at varying degrees of temperature for different lengths of time.

For some years past we have been observing that in our blood-sugar work there is a difference in the results, sometimes quite considerable, if the specimen is examined immediately after collection or if the same is left for varying lengths of time after collection at room temperature. This became more evident when a series of blood-sugar tests has to be carried out throughout the day and night. To find out whether the difference in the results was due to the effect of the temperature, we started keeping all our samples inside a refrigerator at 4°C and found that the difference in the results even if the sample was left for 24 hours was markedly less than if the samples were left at room temperature for 6 hours.

This was really the basis of the present paper which led us to investigate the subject thoroughly, not only from the point of view of glycolysis in samples of blood kept at different temperatures for different lengths of time but also from the point of view of studying the action of any of the different anticoagulants on the rate of glycolysis of blood. To make a preliminary study of the subject we started our work with studies on samples of blood of normal healthy individuals in the following manner —

Oxalated, defibrinated and fluoride samples of blood kept at different temperatures, viz. at 37°C, at room temperature (23°C to 29°C) and at 4°C.

#### COLLECTION OF BLOOD

Blood was collected aseptically by means of a dry syringe. Anticoagulant was used in the proportion of 2 mg per c c of blood. Blood was defibrinated by constant and gentle shaking with broken pieces of glass-beads.

TABLE I

	Average of blood sugar value						Average hourly loss in mg							
	Initial	1st hour	2nd hour	3rd hour	4th hour	5th hour	6th hour	1st hour	2nd hour	3rd hour	4th hour	5th hour	6th hour	Average total loss
At 37°C														
Defibrinated	103.0	86.3	65.2	46.3	35.3	29.5	25.3	16.7	21.1	19.0	11.0	5.8	4.2	77.7
Oxalated	103.4	92.5	82.8	72.0	56.7	41.3	32.1	10.9	9.7	10.8	15.3	13.4	11.2	71.3
Fluoride	103.3	101.0	99.2	95.0	92.4	80.7	94.0	2.3	1.8	3.6	3.2	5.7	2.7	19.3
At room temperature (23°C to 29°C)														
Defibrinated	103.0	89.2	76.0	67.9	56.8	46.0	31.7	13.8	13.2	5.1	11.1	10.8	14.3	71.3
Oxalated	103.4	96.5	91.5	81.5	68.0	59.5	49.9	6.9	5.0	10.0	12.6	9.4	9.6	53.5
Fluoride	103.3	103.2	102.5	100.0	98.5	97.0	94.3	0.1	0.7	2.5	1.5	1.5	2.7	9.0
At 4°C														
Defibrinated	103.0	101.5	97.0	94.0	91.3	85.8	79.0	1.5	4.5	3.0	2.7	5.5	6.8	24.0
Oxalated	103.4	103.0	102.5	101.0	100.0	98.2	95.7	0.4	0.5	1.5	1.0	1.8	2.5	7.7
Fluoride	103.3	103.5	103.8	103.8	103.7	103.7	103.7							

No special aseptic precautions\* were taken to prevent bacterial contamination except that the pipettes used were dried with alcohol-acetone mixture and the flasks in which the blood was collected were previously sterilized

To save space, instead of giving full tables comprising the details of the results, only a synopsis of the results obtained are given above

From the above results, the following important facts become clearly evident —

#### *Glycolysis at 37°C*

1 In defibrinated blood samples the rate of glycolysis was found to be very high during the first 3 hours, resulting in a marked decrease in the original blood-sugar content The rate of glycolysis during the next 3 hours was comparatively slower

2 In the oxalated samples of blood on the other hand the rate of glycolysis was comparatively slower during the first 3 hours and higher during the next 3 hours The net result was therefore that the total loss of sugar in either of these two samples was more or less the same at the end of 6 hours

3 In the fluoride samples of blood, however, the rate of glycolysis was found to be markedly less throughout the whole period of observation as compared with either the defibrinated or the oxalated samples

The average of the total loss of sugar at the end of 6 hours was about —

77.7	mg	per	100	cc	in	defibrinated	blood	
71.3	"	"	"	"	"	oxalated	"	"
19.3	"	"	"	"	"	fluoride	"	"

#### *Glycolysis at room temperature (23°C to 29°C)*

1 In the defibrinated samples of blood the rate of glycolysis was higher than that of the oxalated samples throughout so that at the end of 6 hours the total loss of sugar in the defibrinated samples was definitely more than that of the oxalated The rate of glycolysis appeared to be more or less uniform during most of the period

2 In oxalated samples the rate of glycolysis was slower than that of the defibrinated samples Oxalated samples show a somewhat slower rate of glycolysis during the first 2 hours than that of the subsequent period

3 In fluoride samples of blood the rate of glycolysis as stated before was found to be the lowest

The average of the total loss of sugar at the end of 6 hours was about —

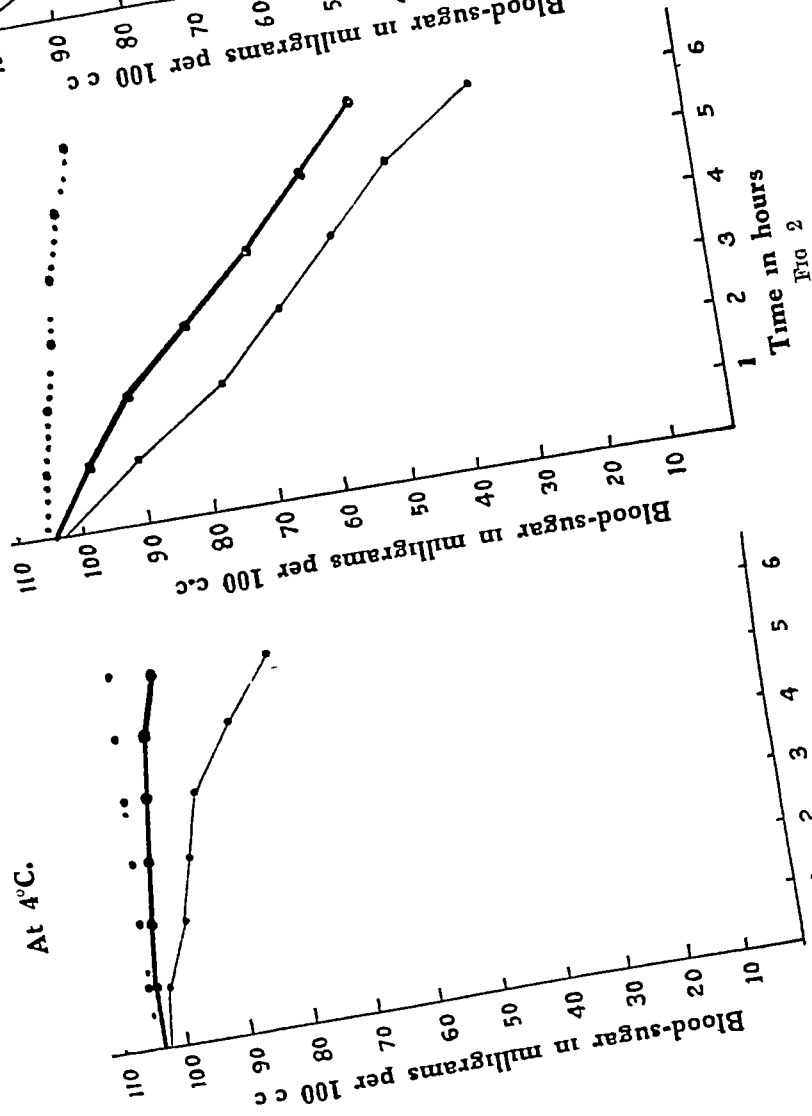
71.3	mg	per	100	cc	in	defibrinated	blood	
53.5	"	"	"	"	"	oxalated	"	"
9.0	"	"	"	"	"	fluoride	"	"

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\* Falcon Lessees (*loc cit*) has shown that aseptic precaution does not alter the result Tolstoi (*loc cit*) also observed that bacterial contamination does not effect the results during the experimental periods of 24 hours

GRAPH 1

At room temperature (23°C to 29°C.)



Key—  
 ..... Fluoride  
 —●— Oxalated  
 — De fibrinated

FIG 1.

At 37°C.

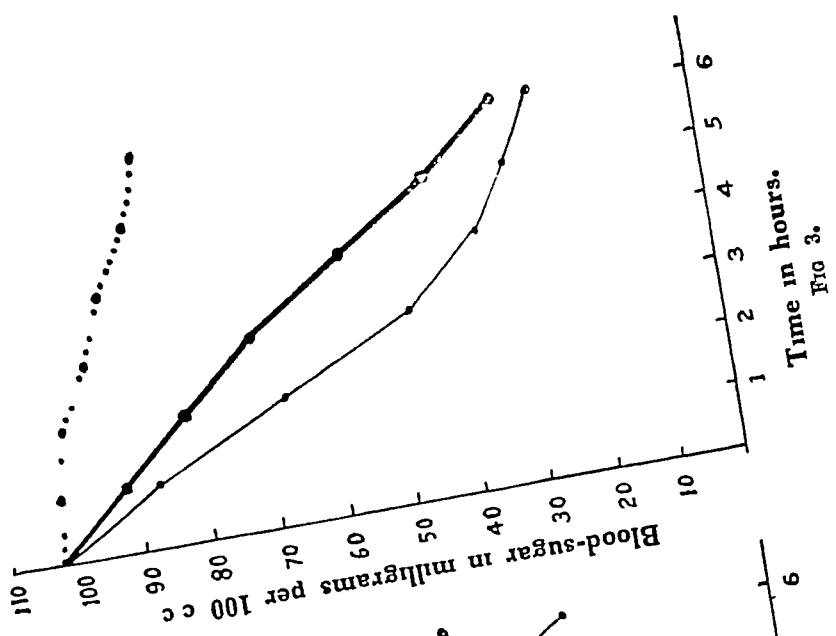


FIG 3.

Key—  
 ..... Fluoride  
 —●— Oxalated  
 — De fibrinated



*Glycolysis at 4°C*

1 In defibrinated samples the rate of glycolysis though markedly depressed than what was observed either at 37°C or at room temperature was, however, still definitely higher than that of the oxalated samples. The rate of glycolysis appears to increase to some extent after 4 hours.

2 In oxalated samples the average rate of glycolysis was, as stated before, definitely slower than that of the defibrinated samples. The rate of glycolysis was, however, more or less uniformly slow throughout.

3 In fluoride samples glycolysis was practically absent\*, the blood-sugar remaining almost the same throughout the period of observation.

The average of the total loss of sugar at the end of 6 hours was about —

24.0 mg per 100 c.c. in defibrinated blood	
7.7 " " " " " oxalated	"
No loss in the fluoride blood	

In order to give a clear exposition of the above results, we have represented them graphically as above.

In order to study this subject more critically we extended the period of our observation from 6 to 24 hours, 48 hours and sometimes up to 72 hours. In this experiment we did not take into consideration the hourly changes in the blood-sugar content as we had hitherto done but only noted the initial blood-sugar level and the changes in the sugar content which took place at the end of 6 hours, 24 hours, 48 hours and 72 hours in a certain number of samples†.

As before, to save space, we represent a synopsis of the results obtained in Table II.

*Glycolysis at 37°C*

A casual observation of the results given in Table II showed that the defibrinated samples of blood lost the greatest amount of sugar at the end of 6 hours at 37°C and the loss thereafter up to 24 or even 48 hours was practically negligible. It also appeared that the defibrinated samples of blood at 37°C lost nearly the whole of its sugar content at the end of 24 hours and that there was no further loss even when blood samples were kept for another 24 hours.

In the case of oxalated samples of blood at 37°C the loss of sugar was also almost as rapid as in defibrinated blood, the major portion of the sugar content being lost within 6 hours and the loss thereafter was slower but complete at the end of 24 hours.

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\* In a few samples of the fluoride blood an actual increase in the sugar content (instead of decrease) was observed at times. The increase, however, was very small not exceeding 1 mg in one hour.

† We should note here that the observation up to 72 hours could not be continued in all the samples of blood due to the specimens being unsuitable for further work owing to deterioration.

TABLE II

	AVERAGE OF BLOOD SUGAR VALUE					AVERAGE LOSS OF SUGAR IN MG. AT THE END OF				
	Initial	6 hours	24 hours	48 hours	72 hours	6 hours	24 hours	48 hours	72 hours	Average total loss
<i>At 37°C</i>										
Defibrinated	103.0	25.3	23.2	22.7		77.7	2.1	0.5		80.3
Oxalated	103.4	32.1	24.1	22.4		71.3	8.0	1.7		81.0
Fluoride	103.3	84.0	73.4	60.6		19.3	10.6	12.8		42.7
<i>At room temperature (23°C to 29°C)</i>										
Defibrinated	103.0	31.7	23.8	23.4		71.3	7.9	0.4		79.6
Oxalated	103.4	40.0	32.3	25.4	23.6	53.5	17.6	6.9	1.8	79.8
Fluoride	103.3	94.3	92.3	89.0		9.0	2.0	3.3		14.3
<i>At 4°C</i>										
Defibrinated	103.0	79.0	60.6	30.0	26.0	24.0	18.4	30.6	4.0	77.0
Oxalated	103.4	95.7	86.5	66.9	30.2	7.7	9.2	19.6	30.7	73.2
Fluoride	103.3	103.7	103.7	102.9						

The fluoride blood samples at 37°C, however, showed entirely different results in that the loss in the sugar content at the end of 6 hours was markedly less as compared to either the defibrinated or the oxalated samples of blood. The loss of sugar thereafter though it took place at a slower rate, however, continued till the end of 48 hours.

#### *Glycolysis at room temperature*

It will be evident that the loss of sugar in defibrinated blood at room temperature was almost the same as at 37°C.

In the case of oxalated samples the loss of sugar was almost the same as at 37°C with the only difference that there was a slow but continuous loss of sugar up to 48 hours.

In fluoride samples the amount of sugar lost during the first 6 hours was definitely less than at 37°C. The loss of sugar thereafter up to 24 or even 48 hours was practically negligible.

#### *Glycolysis at 4°C*

At 4°C, however, the loss of sugar in defibrinated samples during the first 6 hours was not so rapid as at 37°C or at room temperature. The loss of sugar for the next 18 hours, i.e. from 6 to 24 hours, was very slow, the loss thereafter again was rapid and continued up to the end of 48 hours. The loss of sugar from 48 to 72 hours is practically nil.

The loss of sugar in oxalated samples of blood was very gradual there being a marked contrast with the defibrinated sample at the same temperature up to 48 hours but the loss of sugar from 48 to 72 hours was very rapid and reached almost the same level as in the case of defibrinated blood.

In fluoride samples of blood the results are very striking. Here there was practically no loss of sugar at all even up to the end of 48 hours.

#### DISCUSSION OF RESULTS

From the data we have so far collected, it becomes clearly evident that when a sample of blood is allowed to stand, the sugar gradually disappears from it and that the rate of this disappearance of sugar depends on the method of collection and the temperature at which it is kept. For instance, it will be seen that in the case of defibrinated blood samples, the rate of disappearance of sugar was very quick particularly when the samples were either incubated or left at room temperature. Under such conditions it was found to lose about 70 per cent of its sugar content within a period of 6 hours. Even when the defibrinated blood samples were kept at low temperature the loss of sugar was fairly considerable (above 20 mg in 6 hours).

In the case of oxalated samples at incubation temperature the loss of sugar was almost as rapid as in defibrinated samples. But at room temperature the rate of glycolysis in oxalated samples was slower than that of defibrinated samples for the first 6 hours. The difference between the two samples at 4°C became more marked (being 7.7 mg in oxalated samples as against 24 mg in defibrinated samples).

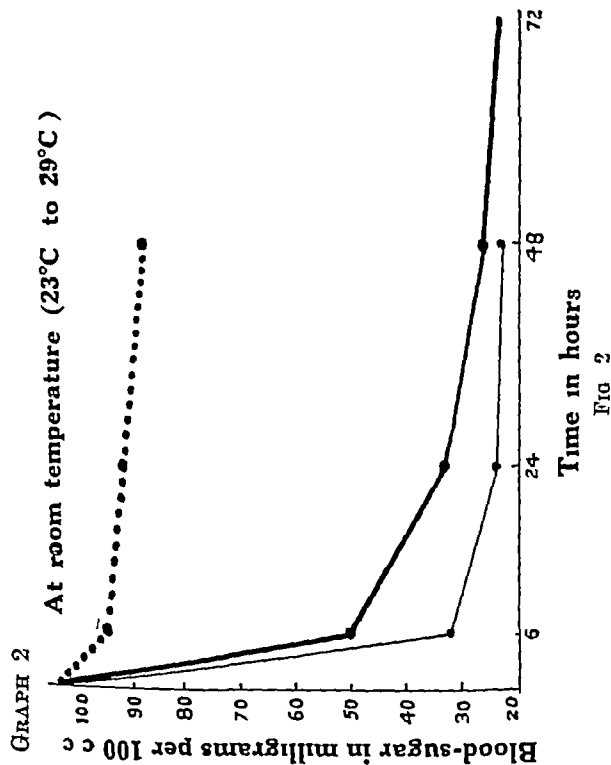


FIG 2

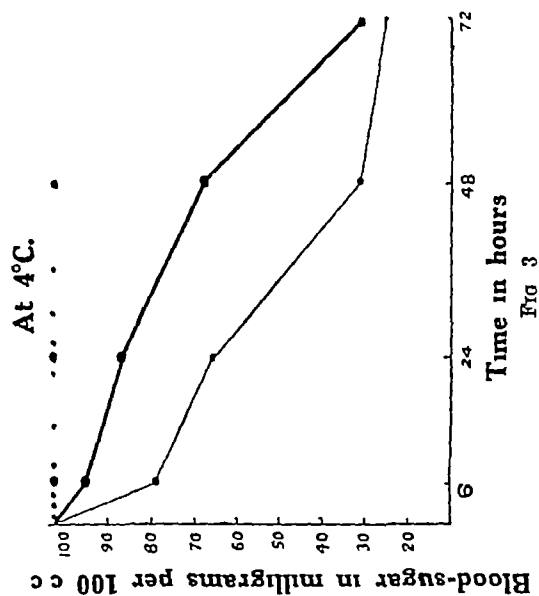


FIG 3

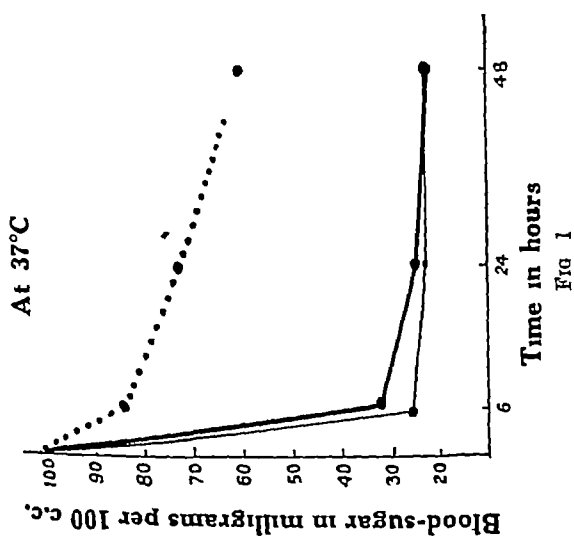


FIG 1

In the case of fluoride samples of blood the loss of sugar at all temperatures was definitely slower than that of either defibrinated or oxalated samples. At 4°C, however, the fluoride samples showed no loss of sugar at all. Further it has been observed that the lower the temperature at which the samples of blood were kept the slower is the rate of loss of sugar.

Apart from the comparative loss of sugar in the different samples of blood at different temperatures, the individual samples with the exception of the fluoride samples, showed rather widely divergent rates of glycolysis at the different temperatures. For instance, as stated before, the defibrinated samples lost 77.7 per cent of sugar at incubation temperature, 71.3 per cent at room temperature and 24 per cent at 4°C within 6 hours. The oxalated samples lost 71.3 per cent at incubation temperature within the same period thus showing little difference with its predecessor as to the rate of glycolysis. At room temperature, however, the loss of sugar in oxalated samples was definitely less (53.5 per cent). At 4°C the oxalated samples lost only 7.7 per cent of sugar which was markedly less than the loss either at 37°C or at room temperature.

The fluoride samples of blood on the other hand showed quite different results, the loss of sugar in these samples being only 19.3 per cent at incubation temperature, 9 mg per cent at room temperature and no loss of sugar at 4°C.

#### TOTAL LOSS OF SUGAR

From the experiments carried out for 24 hours or more it becomes noticeable that the sugar value of some of the samples after coming down to certain limit (20 mg to 30 mg per cent) did not go down any further even if the experiments were prolonged up to 48 or even 72 hours.

Thus, it appears that the maximum reduction of the sugar content in the case of defibrinated samples of blood both at 37°C and at room temperature takes place at the end of 6 hours only\*, after which very slight or no further reduction takes place even if the samples are kept up to 48 hours.

In the case of the oxalated samples, however, at 37°C the maximum loss in the sugar content takes place at the end of 24 hours and if the samples are kept for another 24 hours, there is no further loss. At room temperature though the loss of sugar almost reaches the maximum at the end of 24 hours a slow loss still goes on till 48 hours after which no further loss appears to take place.

We have mentioned above that after the blood-sugar level reaches a maximum low value (between 20 mg and 30 mg per cent range) there is practically no further reduction in the blood-sugar value. We have prolonged the experiment up to 72 hours in a certain number of cases and found that even then there was no further change.

It is difficult to offer suggestions as to the significance of the above findings. It is probable that the sugar content of the blood is capable of reduction up to a

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\* The blood sugar value reaches a limit of 20 mg to 30 mg per 100 c.c.

certain optimum level (20 mg to 30 mg ) beyond which no further reduction takes place It appears that the reducing substance in the blood, which does not show any further change, is not *glucose* but some other non glucose reducing substance such as glutathione and theonine present in the blood There is plenty of evidence in the literature that there is a small fraction of reducing substance present in the blood which has been proved to be non-glucose This substance has been shown to be fairly constant though opinion differs as to the exact amount of this non-glucose reducing substance present in the blood

Somogyi (1927) found the amount of this non-glucose reducing substance in normal blood to be very uniform, averaging 27 mg per 100 c c of blood in terms of glucose

Falcon-Lesses (*loc cit*) also showed that the non-glucose reducing substance in human blood was constant and was 17 mg per 100 c c of blood

De and Bhattacharyya (*loc cit*) observed that the non-glucose reducing substance in normal blood was 6 mg to 8 mg per 100 c c of blood

As we have stated before there is a fairly wide diversity of opinion as to the exact amount of non-glucose reducing substance present in normal blood From the results we have so far obtained it appears to us that the average non-glucose reducing substance present in normal blood is about 25 mg per 100 c c , we are thus inclined to agree with the results obtained by Somogyi (*loc cit*)

#### SUMMARY AND CONCLUSIONS

1 The sugar content of blood undergoes glycolysis if the samples are not examined at once

The rate of glycolysis is variable in different samples and no rate of glycolysis can be predicted

2 The rate of glycolysis depends on the temperature at which the samples are kept and also on the anticoagulant that is used

3 The samples collected without using any anticoagulant (such as defibrinated blood) show the greatest and quickest loss of sugar

4 The rate of glycolysis is definitely high at higher temperatures and low at lower temperatures

5 The rate of glycolysis is high when potassium oxalate is used as an anticoagulant and markedly low when fluoride is used

6 It appears that the anticoagulants have some retarding effect on the glycolysis of blood Of the two anticoagulants we have tried, fluoride to be one of choice for blood-sugar work

7 There is a fairly constant amount of non-glucose reducing substance present in the blood which does not undergo any further change The average amount of this substance present is 25 mg per 100 c c

excitation the stimulating electrode was placed in position before inserting the perfusion cannulae, as the nerves rapidly lose excitability once the perfusion has begun. In all these experiments hypertonic tyrode solution was used (NaCl 0.8 g, KCl 0.02 g, CaCl<sub>2</sub> 0.01 g, MgCl<sub>2</sub> 0.01 g, NaH<sub>2</sub>PO<sub>4</sub> 0.005 g, NaHCO<sub>3</sub> 0.1 g / 80 cc distilled water). Drugs were injected by a syringe in the rubber-tubing connected with the inflow cannula.

#### EFFECT OF NERVE STIMULATION.

1 *The thoracic sympathetic*—Electrical stimulation of the stellate ganglion was done in thirteen experiments. In nine experiments there was a decrease in the rate of flow (Fig 1-a), in three there was no effect and in one experiment, although no effect was seen in the beginning of the experiment, stimulation of the stellate ganglion after injection of 650 $\gamma$  eserine, caused an increase in the rate of flow although eserine by itself had appreciably decreased the flow. It was further noticed that in four experiments previous injection of 250 $\gamma$  ergotamine abolished the effect of stimulation of the stellate ganglion (Fig 1-b). Atropine had no effect on the result of stimulation.

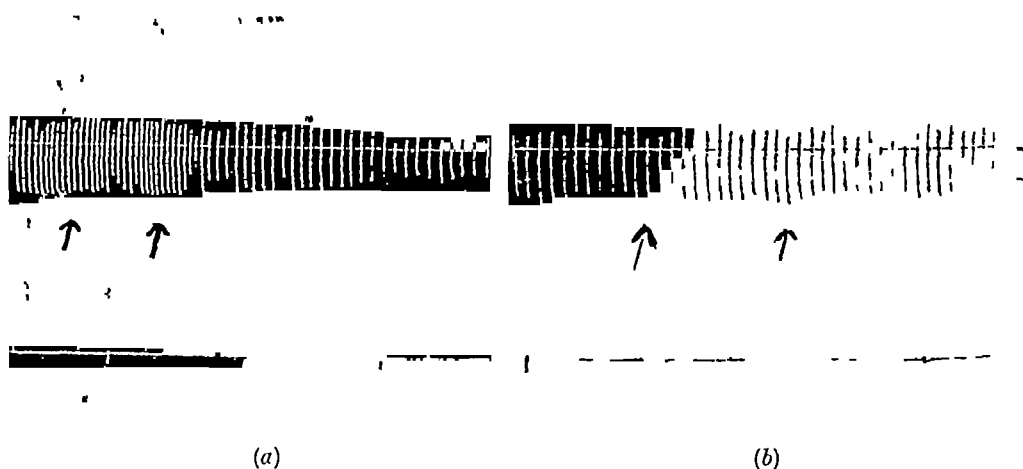


FIG 1

FIG 1—29-4-41—Effect of stimulation of the stellate ganglion on rate of outflow from perfused guinea pig's lungs. Stellate ganglion stimulated between arrows (a) before ergotamine, (b) 8 minutes after injection of 250 $\gamma$  ergotamine. In this and in Figs 2, 3, 4, 5 and 6, upper tracing shows drop record of outflow, lower tracing time in minutes.

The results of stimulation of the stellate ganglion on the pulmonary vascular bed have been variable. Tribe (1914) got either vasoconstriction or vasodilatation or vasoconstriction followed by weak vasodilatation in the cat. Le Blanc and Wijngaarden (1924) got vasoconstriction in the cat. Daly and Euler (1932) got vasoconstriction in the dog. Dale and Narayana (1935) got vasoconstriction in only one out of four experiments in the guinea-pig and they also observed that the effect of stimulation was abolished by previous injection of ergotamine. The present series of experiments go to prove definitely that stimulation of the stellate

ganglion causes pulmonary vasoconstriction and that this effect is abolished by ergotamine. In the one experiment in which vasodilatation was obtained by stimulation of the stellate ganglion after previous injection of eserine, the dilatation was possibly the result of the sympathetic fibres bringing about bronchodilatation which was responsible for the observed vasodilatation. It is natural therefore to conclude that in the thoracic sympathetic there exists vasomotor fibres going to the pulmonary vascular bed. The existence of vasodilator fibres could not be proved.

2 *The vagi*—Electrical stimulation of the vagi was done in seventeen experiments. In thirteen experiments no effect was observed and in four there was definite decrease in the rate of flow showing pulmonary vasoconstriction (Fig 2-a). Injection of even 30 $\gamma$  atropine suppressed this effect (Fig 2-b).

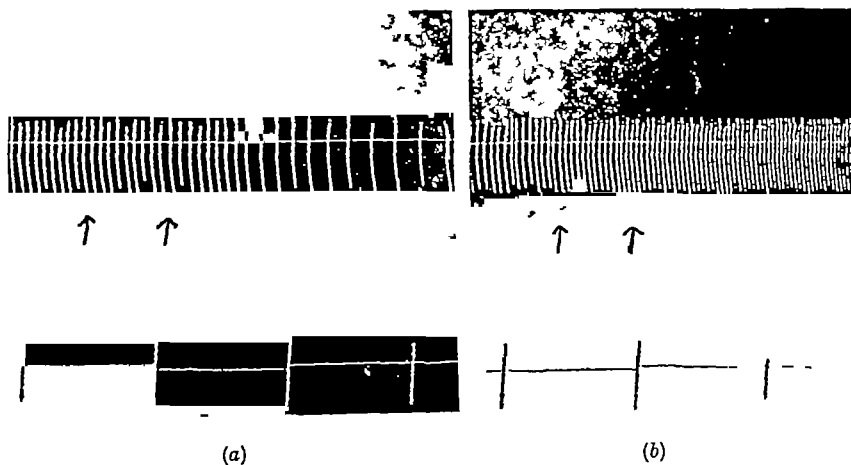


FIG 2

FIG 2—28 3-41—Abolition of vagus effect by atropine. Vagus stimulated between arrows (a) before atropine, (b) 7 minutes after injection of 30 $\gamma$  atropine.

The present work thus gives support to the observation of Dale and Narayana (*loc cit*) on the guinea-pig, of Euler (1932) on the rabbit and of Daly and Euler (*loc cit*) on the dog, and one can conclude that the vagi also carry vasoconstrictor fibres to the pulmonary blood vessels in the guinea-pig. It is, however, possible that under the conditions of the experiment the vasoconstrictor fibres of the vagus ceases to function more quickly than those of the thoracic sympathetic or possibly there also exists vasodilator fibres in the vagus which masks the constrictor effect.

#### ACTION OF DRUGS

1 *Action of adrenaline*—In seven experiments in which adrenaline was injected, six showed a decrease in the number of drops whereas one showed an increase (Fig 3-a). Injection of ergotamine abolished the effect of adrenaline (Fig 3-b).



4 *Action of ergotamine*—Nine experiments were done with ergotamine and it was noticed that in doses ranging from 25 $\gamma$  to 250 $\gamma$  ergotamine showed vasoconstrictor effect in seven and no effect in two (Fig 6)

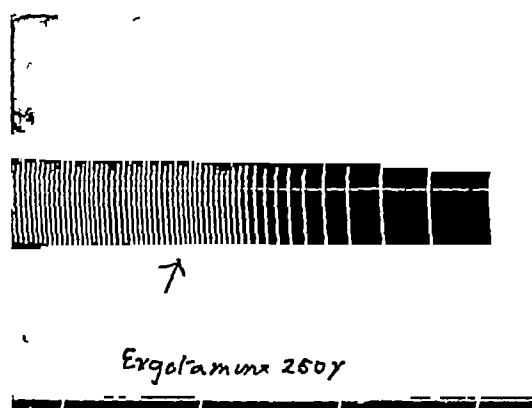


FIG 6

FIG 6—Effect of ergotamine on outflow from perfused guinea pig's lungs 250 $\gamma$  ergotamine injected at arrow

## SUMMARY

- 1 Excitation of the stellate ganglion or injection of adrenaline causes vasoconstriction. In both cases the effect is abolished by ergotamine.
- 2 Excitation of cervical vagi or injection of acetylcholine causes vasoconstriction. In both cases the effect is abolished by atropine and not enhanced by eserine in the case of acetylcholine.
- 3 Injection of either ergotamine or eserine causes vasoconstriction.

## ACKNOWLEDGMENT

I wish to express my thanks to Professor B. Narayana for suggesting this work, for his advice and encouragement throughout and for placing his laboratory facilities at my disposal.

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# A STUDY OF THE SIZE OF HEART IN CASES OF SEVERE ANÆMIA OCCURRING IN THE UNITED PROVINCES OF AGRA AND OUDH IN INDIA

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[Received for publication, August 11, 1941]

## INTRODUCTION

ENLARGEMENT of the heart in cases of severe anæmia has been recognized for many years, but no attempts to confirm the findings radiologically had been made till 1931, when Ball reported his first case of anæmia, due to bleeding fibroids, presenting a big heart, which receded on improvement of anæmia. There was no large series of cases, examined by tele-radiograms, demonstrating cardiac enlargement with subsequent reduction on improvement of anæmia, when these observations were started. Since then, however, one report by Porter and Richmond (1937) has appeared demonstrating cardiac enlargement in cases of ankylostomiasis.

One of the important objects in undertaking this work was to judge precisely the part played by anæmia in producing permanent enlargement of the heart. With this object in view cases have been followed for months after their discharge from the hospital.

## TECHNIQUE

1 *Selection of cases*—Cases of very severe anæmia only, likely to give a total red count of a million r b c per c mm or less, were selected. Ankylostome cases were preferred because they showed anæmia of varying grades of severity, are mostly free from other complications and the anæmia can be rapidly improved. However, other types of anæmia were not excluded and thus the report is fairly representative of the abnormal conditions prevalent in this part of India.

Cases of anæmia with ascites, or those due to nephritis, were excluded. Pregnancy anæmias also were not taken into consideration because in all these

cardiac findings are affected by the displacement of the heart due to distended abdomen

2 *Examination of cases*—A detailed history of the patient was taken on admission inquiring about his family history, habits and past illnesses, particularly malaria and dysentery, and then noting his present complaint with the sequence of events leading to the present condition

A detailed clinical examination of the patient was then undertaken and the necessary laboratory investigations carried out. These primary records were obtained as early as possible mostly within the first three days of admission and later on repeated frequently

(a) *Apex beat*—The apex beat was not recorded in relation to nipple line, but the distance of the apex from the mid-sternal line was measured. The measurement was done tangentially to the anterior chest-wall and not circumferentially around the chest (White, 1937)

(b) *Blood studies*—All blood studies were carried out by using counting chambers and pipettes certified by the United States Bureau of Standards. The hæmoglobin estimations were done with Hellige's hæmometer using Sahli's (1895) standardized scale, so that 14.5 g were equivalent to 100 per cent (Wintrobe, 1930)

The Hellige's instrument was selected because it has non-fading colour prisms and not fluid the tint of which alters with age. The blood studies were always done at eight in the morning before the patients had anything to eat.

The following blood investigations were carried out as a routine and repeated bi-weekly or weekly, certain examinations like reticulocyte count being done daily while the patient was getting vigorous anti-anæmic treatment

- 1 R b c count per c mm
- 2 Hæmoglobin percentage (Hellige's instrument, Sahli's scale)
- 3 Colour index
- 4 Average red cell diameter by Léves' halometer
- 5 W b c count per cm
- 6 Differential w b c count per cm
- 7 Reticulocyte count by cresyl-blue method of staining
- 8 General picture of the blood film

(c) *Examination of stools*—Stools were examined in every case of anæmia and three examinations, including one by concentration flotation method, were done before ruling out the possibility of ankylostome infection

(d) Gastric analysis, van den Bergh's reaction and Wassermann reaction were also done in each case

(e) *The size of the heart* was determined at the beginning and at the end of the period of observation by means of tele-radiograms taken with patient in erect posture. Patient was instructed to take shallow breaths and the operator waited until initial excitation had passed off. The patient was then instructed to hold his breath in mid-inspiration and the plate exposed

The electro-cardiograms were made under standard conditions in the X-ray Department of King George's Hospital with the patient in recumbent position

Transverse diameter was taken as the criterion for the heart size, because the validity of transverse diameter as an index of the size of the heart has been attested by a series of workers Bardeen (1918) Hodges and Eyster (1926), Bedford and Treadgold (1931), Treadgold and Burton (1932) and finally Joseph (1933) have all one after the other shown that transverse diameter of the heart is a true index of its size

### OBSERVATIONS

The following pages contain the various observations presented in a tabular form and illustrated graphically and by tele-radiograms

#### *Size of the heart*

The outstanding feature in almost all cases was the enlargement of the transverse diameter of the heart as compared to predicted normal (the normal transverse diameter for each case was adopted after the prediction tables of Hodges and Eyster, *loc cit*) A study of Table I illustrates this well —

TABLE I

#### *Enlargement of the heart in cases of anaemia*

Reference number	Transverse diameter, cm.	Predicted normal transverse diameter, cm	Enlargement, cm	Type of anaemia	Hb, per cent
1	13.6	10.9	2.7	Hyperchromic	18
2	17.1	11.0	6.1	"	11
3	16.6	11.4	5.2	Hypochromic	23
4	17.1	11.1	6.0	Hyperchromic	20
5	14.3	10.8	3.5	,	23
6	14.7	11.0	3.7	"	16
7	15.0	11.4	3.6	Hypochromic	14

TABLE I—concl'd

Reference number	Transverse diameter, cm	Predicted normal transverse diameter, cm	Enlargement, cm	Type of anæmia	Hb, per cent
8	13.2	11.2	2.0	Hypochromic	35
9	13.2	11.1	2.1	"	28
10	14.4	11.0	3.4	Hyperchromic	13
11	14.8	11.4	3.4	"	11
12	12.1	10.7	1.4	"	30
14	13.3	11.3	2.0	Hypochromic	27
15	13.7	11.6	2.1	Hyperchromic	52
16	11.7	10.7	1.0	Hypochromic	25
17	12.7	10.8	1.9	"	13
18	12.4	10.9	1.5	"	20
19	12.9	10.8	2.1	"	22
20	11.6	11.2	0.4	Hyperchromic	45
21	13.5	11.2	2.3	"	59
22	12.9	11.0	1.9	"	22
23	13.3	11.0	2.3	Hypochromic	31
24	14.7	11.3	3.4	Hyperchromic	44
25	12.6	11.0	1.6	Hypochromic	14
26	11.6	11.2	0.4	Hyperchromic	42
27	13.6	10.8	2.8	"	27
28	8.1	10.9	2.8 less	Hypochromic	60
29	13.9	11.0	2.9	"	14
30	14.8	11.4	3.4	"	18
31	11.2	Data incomplete		Hyperchromic	18
32	9.2	10.6	1.4 less	Hypochromic	29
33	9.9	Data incomplete		"	43
34	10.8	10.5	0.3	"	43
35	8.7	Data incomplete		Hyperchromic	65

*Note*—In calculating the transverse diameter from tables of Hodges and Eyster when—

- (a) weight of the patient was slightly less than 50 kg, the transverse diameter has been calculated on the basis of 50 kg,
- (b) height of the patient was slightly less than 150 cm., the transverse diameter has been calculated on the basis of 150 cm., and when
- (c) both weight and height were below 50 kg and 150 cm respectively, no reading has been recorded

(i) Table I distinctly shows that, with the exception of cases 28 and 32, no case of anæmia showed a normal transverse diameter of the heart. The enlargement varied from a small enlargement of 0.3 cm (case 34) to a great enlargement of 6.1 cm in one (case 2) and 6 cm in another (case 4).

(ii) The other finding that is apparent, on a closer examination of the table, is the variation in the grade of enlargement in different types of anæmia. The cases of hyperchromic group show a distinctly greater enlargement in the transverse diameter of the heart than the cases of hypochromic group. This fact which had so far not attracted attention is well illustrated by the following tables and graphs —

TABLE II

*Percentage of cases of different types of anæmia showing enlargement of various grades*

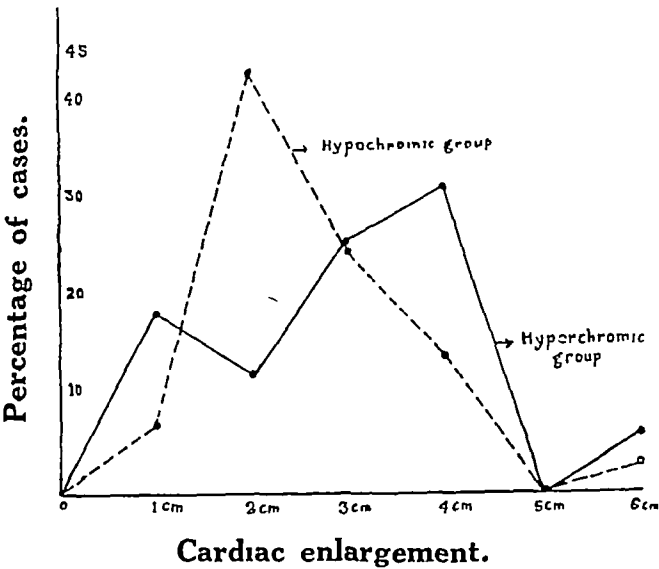
Amount of increase in transverse diameter, cm.	Percentage of hyperchromic group	Percentage of hypochromic group
0-1	18.8	7.0
1-2	12.2	42.9
2-3	25.8	25.0
3-4	31.3	14.3

Thus, we find that maximum number of cases, i.e. 42.9 per cent in hypochromic group, show an enlargement between 1 cm and 2 cm, while the maximum number of cases, i.e. 31.3 per cent in hyperchromic, show an enlargement between 3 cm to 4 cm and another 25.8 per cent between 2 cm and 3 cm. This shows that greater enlargement of the heart is more commonly found in hyperchromic group.

Graph 1 illustrates the above findings —

GRAPH 1

*Comparison of cardiac enlargement in hyperchromic and hypochromic group of anæmias*



*Relation of cardiac enlargement to percentage of hæmoglobin*

Forman and Daniels (1930) were amongst the earliest workers to have observed the relation between Hb percentage and the size of the heart. They observed, while studying the effects of certain foods on anæmia in rats, that the hearts of those with low hæmoglobin values were considerably larger than the hearts of normal animals. In the present series of observations it has been noticed that in human beings as well there is a definite relation between the size of the heart and hæmoglobin percentage as shown in Table III —

TABLE III

*Relation between hæmoglobin percentage and cardiac enlargement*

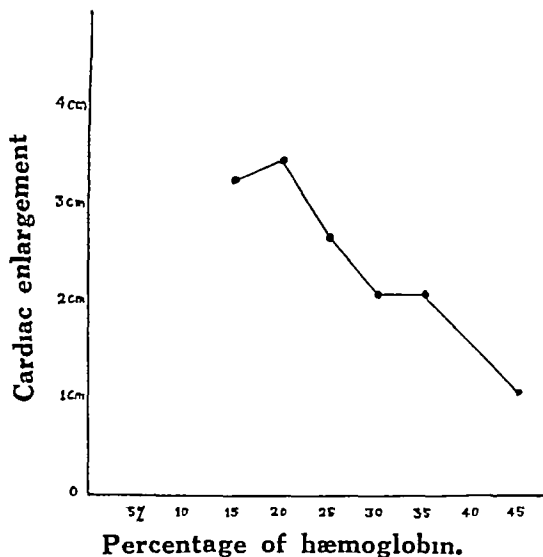
Between 10 and 15 per cent hæmoglobin, the increase is 3.3 cm on an average							
"	16	"	20	,	"	"	3.5 "
"	21	"	25	,	"	"	2.7 "
"	26	"	30	,	"	"	2.1 "
"	31	"	35	"	,	"	2.1 "
"	36	"	45	,	"	"	1.1 "

In other words, there is an inverse relation between the hæmoglobin percentage and the enlargement in the size of the heart

The above findings may be represented as given in Graph 2 —

GRAPH 2

*Relation between percentage of hæmoglobin and cardiac enlargement*



Amongst hyperchromic group of anæmia as well (considering them as a separate class) we find the same relation between hæmoglobin and the size of the heart

TABLE IV.

*Relation between percentage of hæmoglobin and the size of heart in hyperchromic group*

---

In cases between 10 and 15 per cent Hb increase is 4.3 cm on an average

„	,	16	„	20	„	„	4.1	„	„
		21	„	25	„	„	2.7	„	„
„	„	26	„	30	„	„	2.1	„	,
,	,	31	„	45	„	„	1.4	„	,

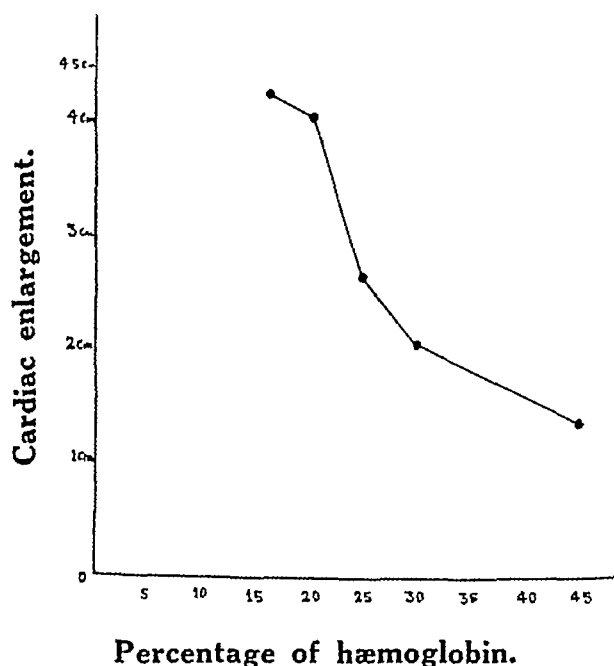
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Below is a graphic representation of the above statement (Graph 3) —

GRAPH 3

*Relation between Hb percentage and the cardiac enlargement in hyperchromic group of anæmia*



*Relation of cardiac enlargement with ankylostome infection*

The cases with ankylostome infection do not present any special feature as regards the enlargement of the heart, as shown below. In fact the enlargement is slightly more marked in non-ankylostome group. The lower grades of enlargement are more common in ankylostome group. 37.5 per cent ankylostome cases show an enlargement between 2 cm and 3 cm as against 14.3 per cent of non-ankylostome cases. But when enlargement above 3 cm is considered, it is found that 42.9 per cent of cases present this big enlargement in non-ankylostome group as against 25 per cent of the other.

TABLE V

*Comparison of cardiac enlargement in ankylostome  
and non-ankylostome cases*

Amount of enlargement, cm	Number of cases	Percentage of ankylostome cases	Number of cases	Percentage of non ankylostome cases
0-1	1	6.3	4	28.6
1-2	5	31.3	2	14.3
2-3	6	37.5	2	14.3
3-4	3	18.7	4	28.6
4-5	0	0	0	0
5-6	1	6.3	2	14.3

Thus, we see that as greater enlargement of the heart is approached, the percentage of non-ankylostome cases who show this preponderates, though when we consider smaller degrees of enlargement, greater number of ankylostome group of cases show it.

*Recession in the transverse diameter of the heart with the improvement of anæmia*

Though the cardiac enlargement in cases of severe anæmia was reported upon by various workers, no one had followed the recession in the size of the heart as the cases improved till 1931, when Ball (*loc cit*) reported his first case of cardiac enlargement due to anæmia with subsequent recession on improvement of blood picture. References in literature on this subject seem to be very scanty even to-day.

This matter has, therefore, been a matter of special inquiry by the writer, and an attempt has been made, in the present series of observations, to record and elucidate the recession in cardiac enlargement as a result of improvement in the anæmia.

Two tele-radiograms showing the cardiac diameter on admission and on discharge are given in Plate I. The figures given are composed of the first and the last tele-radiograms the latter being super-imposed on the former and being indicated by the dotted lines. As some degree of accuracy is sacrificed by the adoption of this method for illustrative purposes, the actual measurements of the original are given below each figure.

The rest of the tele-radiograms are not reproduced because it will take a large space, but the transverse diameter on admission and discharge along with the recession that occurred is given in Table VI.

A study of the preceding tele-radiograms and Table VI will show that cases of *anæmia on improvement of blood picture show a recession in the size of the heart* —

TABLE VI

*Recession in the transverse diameter of the heart  
with the improvement of anæmia*

Reference number	Transverse diameter on admission, cm	Transverse diameter on discharge, cm	Period of observations, days	Recession, cm
1	13.6	13.6	43	<i>Nil</i>
2	17.1	12.4	146	4.7
3	16.6	16.6	31	<i>Nil</i>
4	17.1	16.1	35	1.0
5	14.3	11.9	118	2.4
6	14.7	12.7	111	2.0
10	14.4	13.3	50	1.1
12	12.1	10.9	44	1.2
13	13.2	12.6	26	0.6
14	13.3	13.0	35	0.3
15	13.7	13.0	28	0.7

# PLATE I



Fig 1

Date	R b c per cm	Hb per cent	Colour index	Transverse diameter, cm
19th February	665,000	23	17	143
16th June	2,950,000	85	14	119

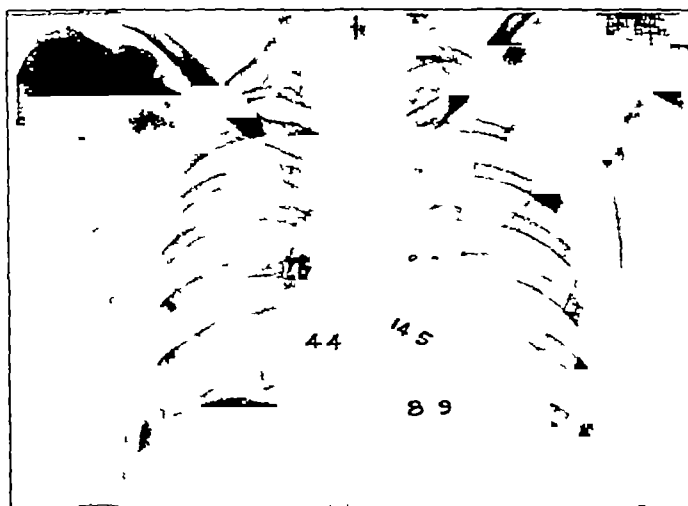


Fig 2

Date	R b c per cm	Hb per cent	Colour index	Transverse diameter cm
17th April	540 000	13	12	144
5th June	3,100,000	72	12	133



TABLE VI—*concl'd*

Reference number	Transverse diameter on admission, cm	Transverse diameter on discharge, cm	Period of observations, days	Recession, cm
16	11.7	11.0	26	0.7
18	12.4	11.6	35	0.8
19	12.9	12.3	37	0.6
20	12.0	11.6	31	0.4
21	13.5	12.6	52	0.9
22	12.9	No improvement, hence not repeated		
25	12.6	12.4	32	0.2
26	11.6	11.3	36	0.3
27	13.6	12.7	36	0.9
28	8.1	Recorded because of small size of the heart		
29	13.9	11.9	21	2.0

Summarizing the above, we find —

	Per cent.
No recession	5.3
Recession between 0.1 cm and 0.5 cm	21.0
"        0.6     "    1.0     "	42.1
"        "    1.1     "    2.0     "	21.0
"        "    2.1     "    3.0     "	5.3
3.1     4.0     "	0
4.1     5.0     "	5.3

Thus, on a survey of Table VI we find that recession in the size of the heart on improvement of anæmia is a fairly constant finding. The majority of cases, 68.4 per cent, show a reduction below 1 cm, about 21.0 per cent between 1 cm and 2 cm and not more than 5.3 per cent 3 cm or over.

## ROLE OF ANÆMIA IN THE ÆTIOLOGY OF HEART FAILURE

On comparison of the size of heart of these cases after improvement of anæmia with the predicted normals for each of these, as is done below, it is found that anæmia plays an important rôle in producing cardiac enlargement

TABLE VII

*Amount of residual enlargement*

Reference number	Age, years	Blood pressure	Predicted normal T D, cm	Actual transverse diameter on relief, cm	Residual enlargement, cm
1	30	102,50	11 2	13 6	2 4
2	28	113,50	11 0	12 4	1 4
3	56	154,66	11 6	16 6	5 0
4	45	104,40	11 4	16 1	4 7
5	26	110,60	10 9	11 9	1 0
6	20	120,65	11 0	12 7	1 7
10	27	108,55	11 3	13 3	2 0
12	21	120,50	11 0	10 9	0 1
13	47	136,85	11 3	12 6	1 3
14	28	120,65	11 6	13 0	1 4
15	42	130,82	11 6	13 0	1 4
16	13	136,70	10 8	11 0	0 2
18	40	128,78	10 9	11 6	0 7
19	20	110,62	11 0	12 3	1 3
20	30	110,75	11 4	11 6	0 2
21	31	116,78	11 4	12 6	1 2
25	17	116,45	11 2	12 4	1 2
26	35	95,46	11 5	11 3	<i>Nil</i>
27	27	105,52	11 2	12 7	1 5
29	30	135,68	11 3	11 9	0 6

A survey of Table VII shows that a certain amount of enlargement may persist even after the blood picture has returned to normal.

This suggests that anæmia may be a cause of permanently enlarged hearts

#### DISCUSSION AND REFERENCE TO LITERATURE

Cases of severe anæmia with enlargement of the heart, detected on clinical examination by percussion have been reported in very early literature (Gautier, 1899), but there was no observation except that of Ball (*loc cit*) illustrating the cardiac enlargement in cases of anæmia, with subsequent recession on improvement of the blood picture illustrated by tele-radiography when the present series of observations were started. Ever since then however observations on the size of the heart in ankylostomiasis both on admission and after improvement have been recorded by Porter and Richmond (*loc cit*)

One of the earliest observations on the size of the heart was those of Gautier (*loc cit*) who found cardiac enlargement on percussion in twenty out of twenty-two patients with severe chlorosis

Later on, pernicious anæmia was found to give a large heart. Kraus (1905) found cardiac enlargement on percussion in 30 of his 47 cases of pernicious anæmia. Large hearts unexplained by valvular lesions or hypertension were found by Wallgren (1922) in six out of seven of his cases, and by Cabot (1926) in twenty-two of twenty-three cases of pernicious anæmia. On necropsy, Strieck (1924) had reported that thirty per cent of a series of 165 patients with pernicious anæmia had enlarged hearts. Goldstein and Boas (1927) have analysed their observations at Montefiore Hospital and report that out of 39 patients 23 had enlargement of the heart mostly to the left and occasionally to the right, as determined by percussion. In six cases, the observation of enlargement was verified objectively by tele-radiogram. All the 12 cases which came to necropsy showed cardiac dilatation. The dilatation involved all the chambers of the heart including the pulmonary conus in 4 cases.

In the present series of cases, it has been clearly shown that cases of anæmia do present definitely generalized cardiac enlargement as demonstrated by tele-radiography (Table I) and that this enlargement recedes to a considerable extent on improvement of anæmia (Table VI)

So far there had been no attempts to judge if the hearts in different types of anæmia behaved differently. The above cases were separated into hyperchromic and hypochromic group, and it has been found on analysis that cases of hyperchromic group present a greater degree of cardiac enlargement (Table II)

Regarding the functional efficiency of enlarged hearts if it is agreed that an enlarged heart is a diseased heart, the deduction that the enlarged hearts of those who have suffered from severe anæmia are diseased, will not be far-fetched. It would be interesting to observe how such hearts react to the effects of age and of acute illness

On general principles, it seems logical to assume that such cases have a permanently lowered myocardial efficiency and are, therefore more liable to myocardial failure than healthy hearts



From the figures in Table VII it appears that an absolute return to normal size is exceptional. This may, however, partly be due to the fact that the follow-up work could not be extended to more than a few months, and, therefore, the comparison and contrast could not be made at a stage when health and blood had completely returned to normal. It is, however, possible that in many advanced cases with degenerative changes in the myocardium, persistent residual defects are left in the shape of some cardiac enlargement, and decreased myocardial efficiency.

Further work seems necessary to assess the efficiency of hearts permanently enlarged after anæmia, but in any case such hearts would give rise to the problems of diagnosis.

Regarding the cause of this enlargement, there have been held various views by different workers. Ludke and Schuller (1910) produced enlargement of the heart in dogs by rendering them anæmic, and Forman and Daniels (*loc cit*) while studying the effect of certain foods on anæmia in rats observed that the hearts of those with low hæmoglobin were considerably larger than the hearts of normal animals.

Fahr and Rhonzone (1922) and Bonchut and Froment (1934) attribute cardiac hypertrophy to increased amount of work that the heart is called upon to do in anæmias. They maintain that though the viscosity of blood is always lowered and resistance to circulation correspondingly diminished, thus apparently giving less work to the heart, yet it is really not so, because in order to maintain the oxygen exchange despite the enfeebled blood, a greatly increased rate of current is required. This must lead to tachycardia and with it the diastolic size of the heart must increase. Thus, both tachycardia and enlargement of the heart are usually found in anæmia. Porter and Richmond (*loc cit*) have described this enlargement in two stages, the first being physiological enlargement, which, if anæmia persists passes into the second stage of hypertrophy of the myocardium. The size in the first stage is reducible, while from the second stage it recedes but little.

Lewis and Durr (1923) and Goldstein and Boas (*loc cit*) attribute this enlargement of the heart to insufficient blood supply to the organ.

It is difficult to say to what extent the two factors, i.e. dilatation due to insufficient blood supply to the myocardium and hypertrophy consequent upon excessive work, are responsible for the cardiac enlargement, because very few cases of anæmia treated on modern lines come to the post-mortem table.

However, it seems evident that dilatation must play the major part, because hypertrophy alone, however marked, is not likely to give an increase of 4 cm. to 6 cm. in the diameters of the heart, and if once this change had occurred, it would not be a reversible one.

In the present series, in a fair number of cases, the heart was bigger than normal by 3 cm. to 5 cm., and in most of these, the size receded considerably on improvement of the anæmia, thus showing that dilatation and not hypertrophy was the original change.

## SUMMARY AND CONCLUSIONS

Cases of severe anæmia occurring in this province were investigated from the point of view of cardiac findings particularly the size of the heart. Cases were first examined clinically in detail, and later on subjected to tele-radiography and electro-cardiography. Important laboratory investigations like blood count, stools examination gastric analysis etc., were also carried out.

The anæmia was then removed by appropriate treatment and detailed examination as above carried out again and the two findings compared. They were then asked to report themselves and thus cases were followed for 5 to 6 months. The following conclusions have been drawn —

- 1 That cases of severe anæmia with as low a count as half a million red cells and 11 per cent hæmoglobin are frequently met with
- 2 That a fair number of these belong to hyperchromic group, but are not cases of pernicious anæmia
- 3 That a vast majority of these cases of severe anæmia present cardiac enlargement as detected by tele-radiography
- 4 That cardiac enlargement is more marked in hyperchromic group
- 5 That there is a considerable recession in the size of the heart, as demonstrated by tele-radiography, on improvement of anæmia
- 6 That the size of the heart after a severe attack of anæmia does not return to normal for many months raising the question of whether it ever does so. Thus, anæmia may leave a permanently large heart
- 7 It is suggested, therefore, that such cases which have had severe anæmia may be more liable to suffer cardiac defeat from causes which would have been successfully combated by a normal heart
- 8 That the enlargement of heart is most probably due to dilatation consequent upon deficient oxygen supply to the myocardium. Hypertrophy due to increased work also plays some part but does not seem to be the most important factor

## ACKNOWLEDGMENTS

I am highly indebted to Major R. D. Alexander, I M S, late Professor of Medicine and Physician to King George's Hospital, Lucknow who very kindly spared his precious time to direct me in the work and check the findings.

My thanks are also due to Colonel H. Stott, I M S, formerly Professor of Pathology and Dean of Faculty of Medicine, King George's Medical College, Lucknow, for his very kindly providing me with a research laboratory, and to Dr R. Lal, Radiologist to King George's Hospital, Lucknow, for his kindly giving me all facilities for x-ray examinations.

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# INVESTIGATIONS INTO THE EPIDEMIOLOGY OF EPIDEMIC DROPSY

## Part XV

### INCIDENCE BY SEASON

BY

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*(An Inquiry under the Indian Research Fund Association)*

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[Received for publication, September 9, 1941]

MANY acute epidemic diseases have more or less definite seasonal incidence. In some cases, it is very marked, e.g. in diphtheria and malaria, while in others, it is relatively ill-defined, e.g. influenza. Oftentimes the specific seasonal curve considerably varies from place to place, e.g. cholera. An examination of these epidemiological phenomena, in the light of the ætiology of the diseases, frequently offers a reasonable explanation but in other instances the reason for variation still remains undiscovered, at any rate, an attempt to find an explanation is generally helpful to a better understanding of the ætiological factors.

It is a matter of general belief in Bengal that epidemic dropsy breaks out in an epidemic form during and immediately following the rains. The following list provides such examples from published literature —

Time of incidence	Observer and year	Place of incidence
June-July	Banerji (1937)	Cawnpore
June-August	Anderson (1908)	Comilla jail
July	Brahmachari and Thakur (1926)	Calcutta
July-August	Dalal (1929)	Rangoon
Rainy season	McConnell (1879)	Calcutta
Majority in rainy season	Greig (1912)	Calcutta
July-November	Mukherji (1930)	Burdwan
During and after rains	Megaw <i>et al</i> (1928)	Calcutta, Howrah, Jessore and Sandwip
Mostly during and after rains	Chopra and Bhattacharjee (1935)	Carmichael Hospital, Calcutta
August-September	Nairne (1879)	South Sylhet
August-September	Anderson (1927)	Kalna
August-November	Megaw (1910)	Presidency General Hospital Calcutta
September	Caley (1878)	Mayo Hospital, Calcutta
September	Munro (1908)	Darjeeling
September	Sarkar and Gupta (1927)	Sandwip
September-October	Daley (1908)	Alipur Reformatory School

However there are other instances where outbreaks some of which were of considerable size have occurred during the winter or spring. Of these the following examples may be quoted —

Time of incidence	Observer and year	Place of incidence
October	McLeod (1893)	Shillong
October-December	Banerjee (1929)	Burghum
October-April	O'Brien (1879)	Shillong
November-December	Sarkar (1915)	Muragacha
November-December	McLeod (1881)	Calcutta
November-March	Brahmachari (1923)	Krishnanagar
December-January	Bhowmic and Sarkar (1920)	Malda jail
Cold season	Dutt (1924)	Bastee and Malda jail
Cold season	McLeod (1893)	Sylhet
Cold season	McLeod (1893)	Calcutta
Cold season	Greig (1912)	Calcutta (1878-79)
Larger number of cases between January and March	Dutt (1936)	Backergunj District
January-April	Banerjee (1928)	Allahabad
February	McLeod (1893)	Dacca.
February-April	Ghosh (1928)	Allahabad
April	Sarkar (1915)	Dhanbad

Chopra and Bhattacharjee (1935) have drawn attention to the seasonal incidence of the disease. They obtained a unimodal curve commencing its ascent in July, reaching the fastigium rapidly in the following month and then sharply declining for a while but later on following a gradual downward course and touching the abscissa in April. This curve more or less represents the experience of the first group. It is based on the number of admissions in the Carmichael Hospital for Tropical Diseases during the period 1922 to 1933. Possibly the hospital admissions are a correct expression of the actual prevalence in Calcutta. However, a closer scrutiny of the data reveals the fact that the big epidemics of the two years 1926 and 1932, which contributed 39 and 23 cases respectively out of a total of 131, largely dominate this experience and determine the particular form of the curve, the contribution of other years, which on an average did not exceed seven, had little influence on the shape of the curve. It was, therefore, considered desirable to investigate further the nature of seasonal variation of the disease in the light of the knowledge now available.

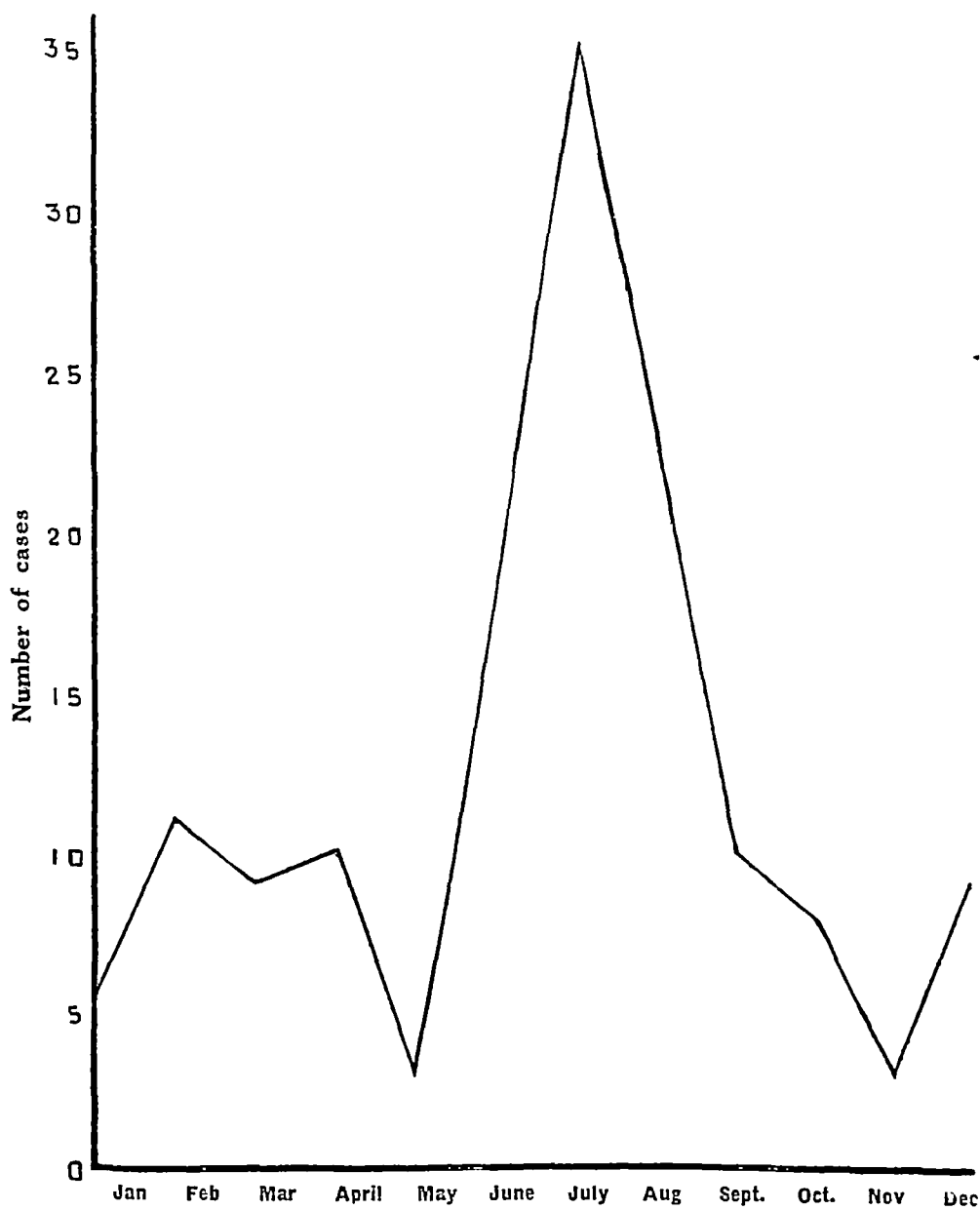
In Graph 1 are depicted the number of cases admitted in the same hospital during the period January 1934 to 1940 according to the month of attack. It will be seen that this curve, which is based on a total of 144 cases, is similar to the curve described by Chopra and Bhattacharjee (*loc cit*) but differs from it in minor details. Thus, the ascent begins a month earlier, the fastigium is also reached a month earlier, the decline is more rapid, there is a small second peak in February and at no time does the curve touch the abscissa. This experience includes a year (1935) of high incidence (50) but no year or years dominate the picture to the same extent as in Chopra's data, because the incidence in four of the remaining six years is not so low and the experience of individual years is more or less similar to that of 1935 except that the vernal rise is more pronounced.

In Graph 2 are presented two curves based on the combined records of published reports. The continuous line represents seasonal incidence of cases and the broken line the number of outbreaks having their peak in the respective months. In some of the reports, only an approximate period such as a quarter of the year is given and these had to be excluded. It will be seen that there is a remarkable similarity between the seasonal case curves in Graphs 1 and 2. The correspondence in the two curves of Graph 2 suggests that as a rule the frequency of epidemic peaks follows the morbidity curve but epidemics of greater magnitude occur during the rains than during the spring.

Data from another source have also been used for drawing a seasonal curve of morbidity (Graph 3), namely the reports of cases received from various health authorities in Bengal in response to a circular requesting the supply of mustard oil samples together with certain epidemiological details. The general shape of the seasonal incidence curve is similar to that of corresponding curves of Graphs 1 and 2, except that the ascent commences earlier and the secondary rise is relatively small. There is in this case a very close correspondence between the morbidity curve and the curve of frequency of peaks of epidemics. The two curves considered together shows that the data are composed mostly of small outbreaks particularly during the spring.

GRAPH 1

## SEASONAL INCIDENCE OF EPIDEMIC DROPSY

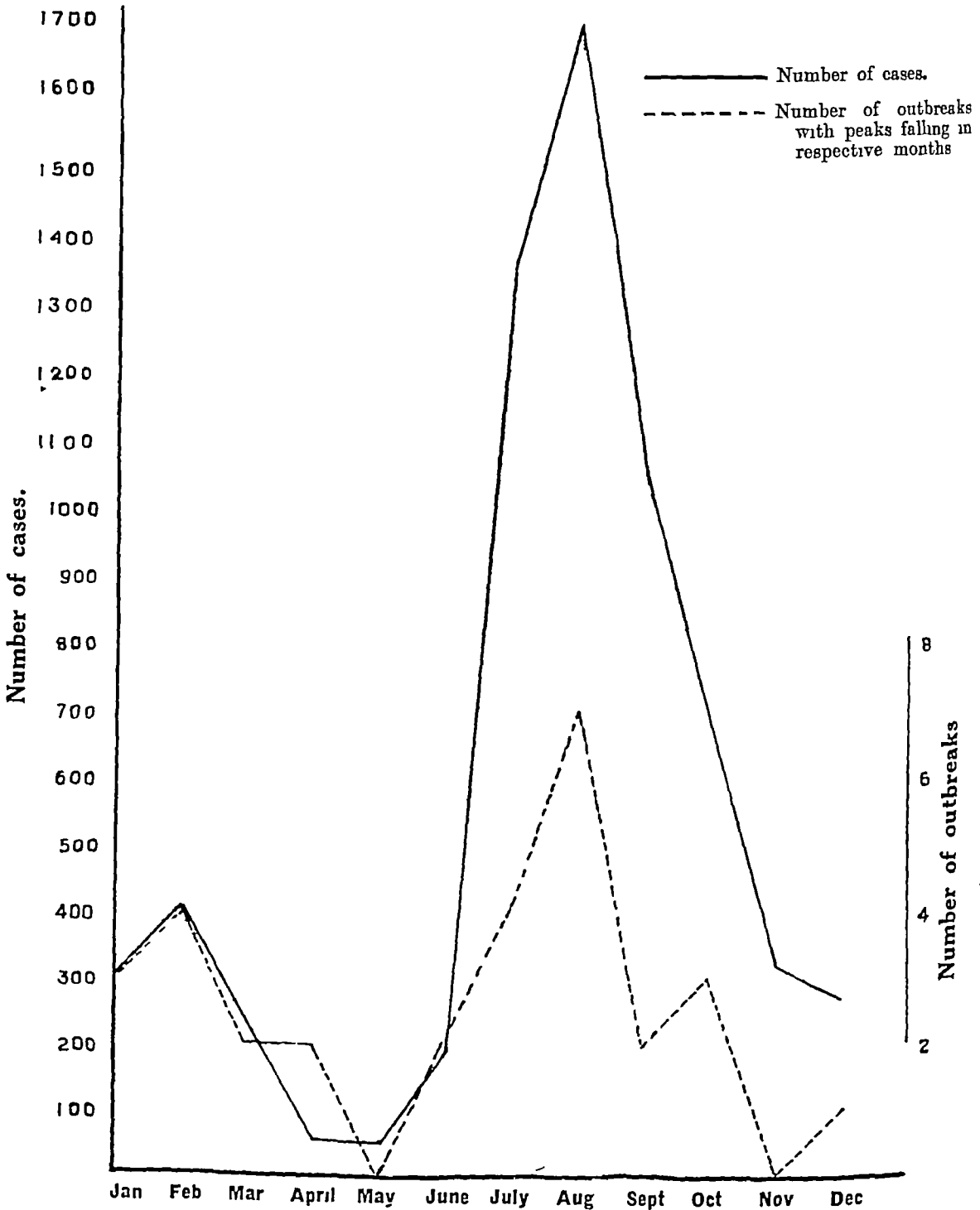
*Cases admitted in Carmichael Hospital for Tropical Diseases, 1934 to 1940 inclusive*



GRAPH 2

SEASONAL INCIDENCE OF EPIDEMIC DROPSY CASES AND OF  
PEAKS OF OUTBREAKS

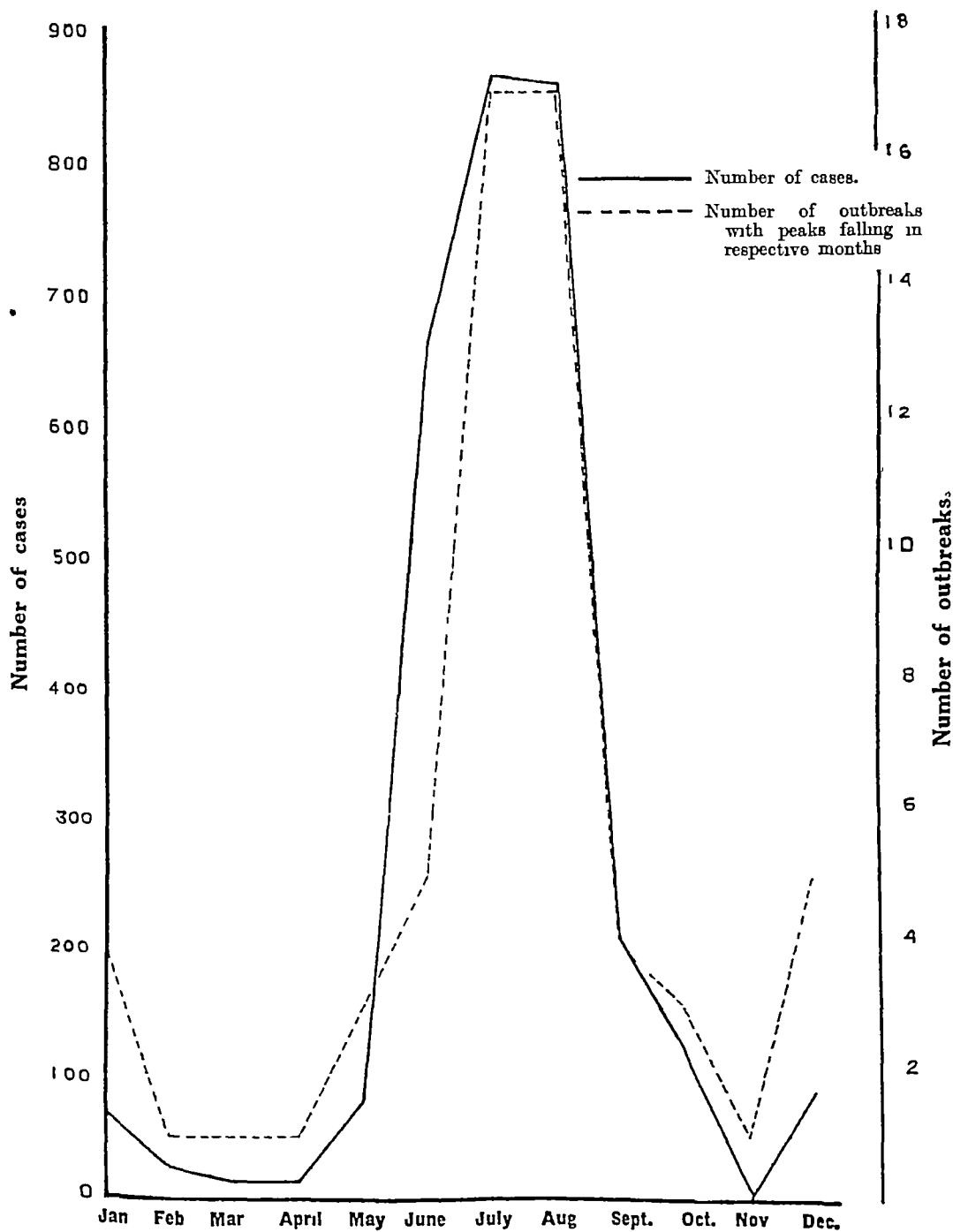
*Combined data of outbreaks described in literature*



GRAPH 3

# SEASONAL INCIDENCE OF EPIDEMIC DROPSY CASES AND OF PEAKS OF OUTBREAKS

*Combined data of outbreaks reported by the local health authorities*

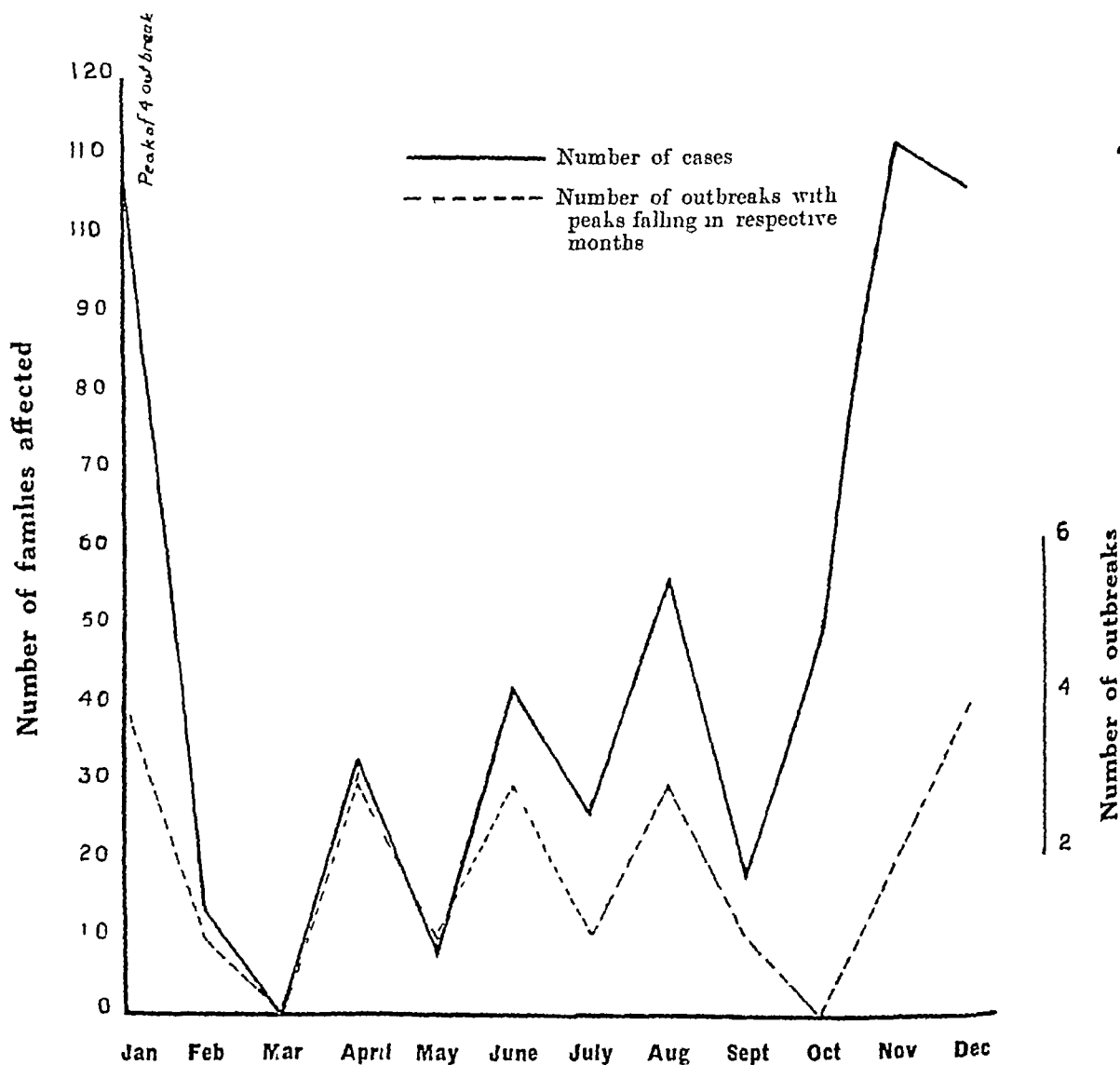


In the course of our investigations on the ætiology of the disease we had occasion to study 23 epidemics of varying sizes in different places in Bengal and the

GRAPH 4

# SEASONAL INCIDENCE OF EPIDEMIC DROPSY CASES AND OF FAMILIES AFFECTED

*Combined data of outbreaks investigated by the authors and colleagues*



neighbouring provinces The seasonal experience of our cases and of the frequency of peaks of epidemics are presented in Graph 4 These curves are entirely different

from those described previously. This departure from the general experience and the irregularity of the curves would suggest that the epidemics studied by us were not a random sample of the epidemics in general. However these curves are of interest in showing that outbreaks of the disease may occur at any time of the year and the off-season epidemics may at times be of considerable size.

From the above data it may be justifiable to conclude that epidemic dropsy is subject to a definite seasonal incidence in Bengal and the neighbouring provinces and Graph 2 may be accepted as the typical seasonal incidence curve. The question may now be asked, what explanation, if any, does the *Argemone mexicana* contamination theory offer for this seasonal distribution? Both the mustard and the *Argemone mexicana* plants are annuals. The crop of the former is gathered during March, the seeds of the latter ripen in most parts about the same time or a little later. New mustard seed appears in the market about April. Allowing a month or two for crushing of seeds and for the manufacture and distribution of the new oil to consumers and an interval of two or three weeks for the incubation period, the main seasonal rise in July–August would appear to correspond with the consumption of the new oil. Though it is a pure speculation, it is possible that the *Argemone* seeds being less plentiful later on, the contamination of the mustard seeds may be less frequent. Besides it is known that the adulterated oil loses its potency on exposure to light and air (*vide* Lal *et al* 1940 1941) to which it is subjected in the retail shops. The secondary rise in the spring cannot be easily explained. Is it due to relapses occurring on account of the physiological state of the body favourable for the appearance of this disease syndrome? That this may be a possibility is suggested by the observation that the crops of cases in the spring frequently follow a sharp outbreak during the rains in the preceding year.

#### ACKNOWLEDGMENT

We are grateful to Brevet-Colonel Sir Ram Nath Chopra, I.M.S. (*Retd*), Director, School of Tropical Medicine, Calcutta, for permission to make use of the records of the Carmichael Hospital for Tropical Diseases.

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## CANNABIS SATIVA IN RELATION TO MENTAL DISEASES AND CRIME IN INDIA

BY

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[Received for publication, September 30, 1941]

### INTRODUCTION

IN spite of the popular belief that excessive and prolonged indulgence in hemp drugs leads to certain forms of mental disorder and crime of a violent nature, little attempt has so far been made to study this question from its scientific aspects. The conclusions of the Indian Hemp Drug Commission (1893-94) were at variance with the popular belief. The Commission considered that 'Moderate use of these drugs produces no injurious effects except in persons with specially marked neurotic diathesis. Excessive use indicates and intensifies mental instability. Moderate use produces no moral injury whatsoever'. Ewens (1904) found that the form of mental disease classed in this country as 'toxic insanity' has a direct relation to excessive use of hemp drugs. Robertson-Milne (1906) and Dhunjibhoy (1927) also came to similar conclusions. None of these workers, however, studied the bearing of these drugs on the prevalence of crime. In this paper an attempt has been made to discuss the part played by poisons in general, and hemp drugs in particular, in producing mental instability and crime in India.

Besides the careful studies made on 1,500 cases of hemp drug addiction by the authors (Chopra and Chopra, 1939), investigations were carried out in almost all the mental hospitals of India, where inmates suffering from toxic insanity were examined from time to time and kept under observation for a number of years. The histories of most of the discharged cases of toxic insanity were also studied.

The cases in which hemp drugs were the apparent cause of insanity were studied individually and attempts were made to obtain all possible information with a view to determining how far the facts elicited were reasonably correct. All doubtful cases were eliminated. In this way it was possible to collect reliable data concerning 600 cases of hemp drug insanity.

#### PHYSIOLOGICAL AND PATHOLOGICAL CHANGES IN HEMP DRUG INTOXICATION

Chopra and Chopra (*loc cit*) studied the effects of hemp drugs on the central nervous system. Since then further work has been done in this connection. There is little doubt that *Cannabis sativa*, like other intoxicants or nerve poisons, may give rise to an altered state of mind which in certain cases may simulate mania. This drug is known to have a marked effect upon the intellectual processes which become irregular, or even partially or totally suspended. *En passant* it may be added that the higher centres, which are late in developing, are more sensitive and more prone to be influenced by narcotic drugs.

The effects upon the brain may manifest themselves in the following ways —

(i) *By causing certain general effects* — These are more or less diffuse in nature and produce vague symptoms, which are common to all intoxicants, e.g. euphoria, dizziness, exhilaration and a feeling of relief from worry, strain, etc.

(ii) *Through their localized or specific action* — Localized or specialized effects of drugs exhibit themselves in the case of special centres in the brain and are believed to differ in the case of each drug. Thus, each narcotic drug is apt to cause certain special symptoms or derangement of mental faculties, in addition to the general effects, which are peculiar to it.

It is apparent, therefore, that in spite of the great dissimilarity in the effects produced by substances which are capable of producing narcotic effects, there are certain clinical features which are common to all. For this reason it is almost impossible to give a definite picture or symptomatology of toxic insanity for each individual drug. Mental derangement resulting from the use of toxic substances is nothing but the reaction on the part of cerebral cells whose regular activity is either held in abeyance or is perverted partially or totally. There is a relaxation of control of the higher centres which allows the lower centres to come under the influence of external abnormal excitations which may be different from the usual stimulation. This reaction may be observed in one or more parts of the central nervous system, leading to differences in the symptoms produced. Thus, it appears that a drug when it is absorbed in the system does not add any new element to the brain. Therefore, a drug simply excites or depresses the existing trend of the mind or mental aberrations if any. Therefore, all types of insanity resulting from intoxication through any drug whatsoever, are characterized by certain general symptoms, such as excitation of the organs of thought, sense of intoxication and incoherent ideas and actions. The symptoms in case of a particular drug may differ widely in individuals. Persons with psychopathic and neurotic tendencies appear to be more easily affected than normal healthy individuals with a stable nervous system.

It is well known that the same dose of a narcotic, for example alcohol, bhang or opium, does not produce similar effects in all individuals. The individual personality and idiosyncrasy of different persons are important factors in the resultant effects. The authors found that the hallucinations occurring in Western people could not be observed in Indians who are more prone to dreams pertaining to sex and of a voluptuous nature. The symptoms may thus be influenced primarily by the selective affinity on the part of special group of cells in the brain for a particular drug and secondarily by the personality of the individual taking the drug.

Besides the above two there are other factors of importance which have to be considered in evaluating the effects of hemp drugs on an individual. They are the degree of education, reasoning, judgment, dosage, mode and time of administration of the drug, etc. All these factors may modify the symptoms and effects. The influences of these factors are discussed in the section on analytical studies. As regards the dosage and the mode of administration, the effects are intense with larger doses and appear much earlier when the drug is taken on an empty stomach. Similarly, different preparations, such as ganja, charas and siddhi (bhanga) give rise to effects which vary a great deal in intensity according to the amount of the active principle contained in them (Chopra and Chopra, *loc cit*).

#### ANALYTICAL STUDIES OF CASES

A survey was carried out on the toxic insanity cases in all the important Indian Mental Hospitals between 1928 and 1939. During this period frequent visits were paid to these institutions and many cases were examined from time to time and individually studied. Keeping in view the physiological and pathological changes and the symptoms observed and those already on record in the history sheets we have made an attempt to discuss the mental disorders resulting from the use of hemp drugs in the following order. Table I shows the results of analysis of 600 cases of toxic insanity with a definite history of indulgence in the use of hemp drugs —

TABLE I

*Giving the diagnosis of 600 cases of toxic insanity studied,  
with history of hemp drug addiction or indulgence*

Type of mental disorder	Number	Percentage
(a) Acute disorders —		
1 Acute confusional insanity mania of incoherent type	200	33.3
2 Maniacal condition of expansive form	45	7.5
3 Toxic hallucinatory disorders	75	12.5



TABLE I—*concl'd*

Type of mental disorder	Number	Percentage
<i>(a) Acute disorders—concl'd</i>		
4 Melancholia	70	11.66
5 Depressive mania	10	1.66
6 Recurrent toxic mania	60	10.0
<i>(b) Chronic disorders—</i>		
1 Chronic toxic mania	90	15.0
2 Schizophrenia	30	5.0
3 Dementia (secondary to hemp drugs)	20	3.33

It is evident from Table I that acute mental disorders from hemp drug habit are much more frequent than chronic conditions

1 *Acute disorders*—Most of the narcotic poisons and especially hemp drugs produce disorders of a temporary nature which disappear without leaving any trace. The effects are limited to slight excitement or depression of faculties, or to a short interval of intoxication and sometimes to profound narcosis. Complete recovery, however, occurs in most of these cases. The duration and nature of symptoms may vary according to the disposition of the individual and the dosage. The acute stage, if recovery should follow, does not last more than a few days or a few weeks. Acute toxic insanity is a secondary insanity which may simulate all forms of insanity. It may differ not only in the case of two different drugs, but also with one and the same drug. It is temporary and may be cured with the elimination of the poison from the body. In this series in 40 per cent of cases the symptoms disappeared within 24 weeks of the onset and in 2.6 per cent within 6 months.

The following conditions deserve special attention—

(a) *Acute confusional insanity*—This condition was most common and probably resulted from a general derangement of the cerebral functions after taking the poison. 37.07 per cent of our cases belonged to this group.

(b) *Maniacal conditions of expansive form*—These were characterized by erotic ideas of self-gratification and exaggeration of personality. This type was found in 7.5 per cent of cases in this series.

(c) *Melancholia and depressive mania*—In this series 11.6 per cent of cases were found to be suffering from melancholia. This condition depends upon the type of individuality and temperament of the addict. Those with sad and morose disposition are more prone to this type of disorder after the use of hemp drugs. A pure melancholia following the use of hemp drugs is rare, but very often the mixed

types of cases which result, are labelled as 'maniacal depressive' In this series 16 per cent of cases belong to this group

(d) *Delusional and hallucinatory type of insanity*—This was present in 12.5 per cent of the cases The delusions were of self-aggrandisement, the tendency to suicide was very rare, but homicidal acts by hemp drug maniacs were not uncommon

2 *Chronic disorders*—Chronic voluntary intoxication may also lead to mental disorders The course of symptoms may depend on the habits of the individual, on the individual's reactions and the dosage The chronic form does not generally establish itself from the very beginning There are at first acute phenomena which may reproduce themselves a number of times without, however, preventing chronicity from establishing itself These acute symptoms are nothing but a recurring picture of the original attack which appears again and again in the course of the disease The two essential kinds of symptoms are the irresistible desire for the poison with the periodical recurrence of the acute or subacute symptoms and the progressive decay of mental faculties The acute symptoms correspond to temporary saturation of the body with the poison, while chronic symptoms are the expression of definite anatomical injury to the brain which gradually results from the effects of the drug The prolonged abuse of the poison thus gives rise to progressive weakening of all the faculties of the brain, eventually leading to dementia In the present series 60 cases or 10 per cent showed relapses (*vide* Table I) and it was found that relapses were more common between the ages of 20 to 30 and 41 to 50 years

(a) *Dementia secondary to hemp drugs*—This condition was rather rare and occurred in only 3.33 per cent of cases in this series

(b) *Schizophrenia*—This was present in 5 per cent of cases

(c) *Chronic toxic mania*—This disorder was found to be much more prevalent than the other two It was present in 15 per cent of our cases

#### ÆTIOLOGICAL FACTORS

These will be considered under three main headings —

- 1 Insanity due to hemp drugs and other correlated factors
- 2 Relationship between occupation and hemp drug insanity
- 3 Age incidence and hemp drug insanity

Investigations on 600 cases of hemp drug insanity from different hospitals revealed that, though it was comparatively easy to elicit the history of hemp drug habit from such patients, it was often difficult to ascertain whether this narcotic was the primary cause of insanity in such cases or indulgence in it was only secondary to the existing mental disorder We have already observed (Chopra and Chopra, *loc cit*) that persons with an unstable nervous system and those suffering from mental disorders are more prone to drug habits and particularly to excesses than normal healthy individuals The former two groups very often indulge in intoxicants

with undue or unwanted freedom, and thereby precipitate the course and aggravate the symptoms of the actual disease. We have therefore rejected all cases with doubtful history and taken into consideration only those cases where a definite authentic history could be obtained. These cases consisted of two main groups —

(1) *Unmixed cases* in which the hemp drug habit was found to be the only elicitable cause of insanity

(2) *Mixed cases* in which other factors such as heredity or indulgence in other intoxicants were also present in addition to indulgence in hemp drug. Table II demonstrates the relative frequency of unmixed and mixed types out of a total number of 600 cases under investigation and shows the various additional contributory factors in the ætiology of mixed types of hemp drug insanity

TABLE II

*Showing factors contributing to the ætiology of 600 cases of pure and mixed hemp drug insanity*

Cases	Number	Percentage
<i>Unmixed cases —</i>		
History of indulging in hemp drugs alone	400	66.6
<i>Mixed cases —</i>		
1 Heredity and hemp drug	15	2.5
2 Heredity and alcohol	10	1.66
3 Heredity, alcohol and business loss	20	3.33
4 Alcohol	30	5.0
5 Alcohol and vice	15	2.5
6 Business losses	10	1.66
7 Syphilis	5	0.83
8 Sexual excess	5	0.83

TABLE II—*concluded*

Cause	Number	Percentage
<i>Mixed cases—concluded</i>		
9 Dhatura	10	1.66
10 Grief	10	1.66
11 Hard life and alcohol	5	0.83
12 Hard life and opium	5	0.83
13 Modak smoking	10	1.66
14 Vicious habits	10	1.66
15 Opium eating	10	1.66
16 Epilepsy	5	0.83
17 Disappointment in love	5	0.83
18 Tuberculosis	10	1.66
19 Miscellaneous	10	1.66

A perusal of Table II shows that out of 600 cases of hemp drug insanity there were 400 cases of unmixed type giving history of indulgence in hemp drugs alone. In the remaining 200 cases which we have placed under the heading of the mixed group, the place of various additional contributory factors has been described. An analysis of these predisposing or determining causes shows that hereditary predisposition was present in 7.5 per cent of cases. This figure seems to be on the low side. It is well known that a narcotic drug even when it is without any action on healthy brains may upset the mental equilibrium in persons born with hereditary predispositions.

Co-association of other intoxicants along with hemp drugs naturally increases the frequency of insanity. In our series history of indulgence in alcohol, dhatura and opium was quite frequent. It is logical to think that the simultaneous use of these narcotics which are also cerebral poisons would further upset the equilibrium by their cumulative effects.

## RELATIONSHIP BETWEEN OCCUPATION AND HEMP DRUG INSANITY

The stress and strain of different occupations is likely to influence the production of insanity amongst the hemp drug addicts. We have therefore investigated this point and this is summarized in Table III —

TABLE III

*Showing the occupational factor in a series of 600 established cases of hemp drug insanity*

Occupations.	Number	Percentage
1 Beggars and sadhus	180	30.0
2 Cultivators	35	5.83
3 Shopkeepers	30	5.0
4 Traders business men	65	10.83
5 Labourers and artisans	60	10.0
6 Domestic servants	45	7.5
7 School teachers	5	0.83
8 Police constables	20	3.33
9 Tailors	5	0.83
10 Students	5	0.83
11 Prostitutes	5	0.83
12 Priests	10	1.66
13 Hackney carriage drivers	10	1.66
14 Peons	5	0.83
15 Clerks	5	0.83
16 Unknown	60	10.0
17 Miscellaneous	55	9.16

From a perusal of Table III it would appear that the largest number i.e. 30.0 per cent occurred amongst the beggars and sadhus who are in the habit of wandering from place to place. It may also be stated that many of these individuals are in the habit of indulging in hemp drugs and often in excess. Next come traders and business men i.e. 10.83 per cent and labourers and artisans 10 per cent. This is

probably on account of the strain and stress of life which members of this group are subject to and which compels them to resort to the use of a narcotic so as temporarily to tide over the period of stress which may lead to mental derangement in predisposed individuals Domestic servants come next in frequency probably on account of an excessive consumption of these drugs

*Age incidence*—Table IV shows the relative liability to insanity at different ages in this series This information was compiled from the history sheets in various Indian Mental Hospitals In a large number of cases the ages entered are only rough guesses on the part of the medical or police authorities It is difficult to ascertain the true ages of patients, especially of those belonging to poor classes, such as beggars and the wandering insane

TABLE IV

*Showing the age incidence in 500  
hemp drug insanes*

Age in years	NUMBER OF CASES	
	First attack.	Second attack or relapse
15 to 20	53	7
21 to 30	252	41
31 to 40	85	7
41 to 50	40	21
51 to 60	15	14
61 and up	5	0
Age unknown	50	0
TOTALS	500	90

A perusal of Table IV will show that the most common age at which an attack is likely to occur is the period of adolescence, i e 15 to 30 years, when the nervous

system is being established, the next common period is between 31 to 40 years after which the incidence falls gradually till it is only 1 per cent after 60 years

### SYMPTOMATOLOGY

*Early symptoms* —As a rule it was difficult to obtain a complete clinical history of the condition of these patients on their first admission. The medical certificates on which the patients were admitted into the hospitals usually contained just sufficient evidence of their mental state to support the diagnosis of insanity. From the examination of a large number of cases and from the hospital records and subsequent entries in the case sheets of these patients it was possible to form an idea of the symptomatology of this form of insanity. Hospital case sheets also dealt with the mode of onset, duration of the attack, the habits and the personal and family history of the patients.

The onset in 40 cases was sudden, in 230 gradual and in 150 insidious. There was no information on this point in the remaining 180 cases. Headache was quite common and was an early complaint, alteration in the habit of the individual also led to suspicion of mental derangement. More frequently attention was drawn by violent or destructive acts which in most of the instances necessitated the removal of the individual to a mental hospital.

*Physical examination* —The state of the general bodily health on admission into the hospital was quite good in 230 cases, in 200 it was fair, in 80 the health was indifferent, in 60 it was bad, while 30 cases were very weak and emaciated. The facial expression in 150 cases was vacant. In the rest no information has been recorded. Four individuals had 'ganja wart' (callosities) on their palms. Abnormalities or signs of disease of the sexual organs were quite frequent.

*Circulatory and respiratory systems* —The circulatory and respiratory systems as a rule showed no abnormality, but signs of anaemia were present in cases where the disease was of long standing.

*Digestive system* —In sixty cases food was refused necessitating forcible feeding. There were no other changes recorded in the digestive system except that at times a few of these patients suffered from attacks of dysentery and diarrhoea. These conditions naturally had no direct relationship to insanity.

*Muscular system* —A large number of the patients suffered from tremors and sometimes from choreiform muscular twitchings. The strength of the hand grip was definitely decreased in almost all the cases.

*Nervous system* —The state of sight, hearing, taste and smell were difficult to investigate. Redness of the conjunctivæ was a very common symptom and has been regarded as pathognomonic of hemp drug insanity by Dhunjibhoy (*loc cit*). Congestion of the transverse ciliary vessels was observed in 70 per cent of the cases. The conditions of nervous sensibility, hyperaesthesia and anaesthesia varied in different individuals. All the reflexes were exaggerated in most of the cases in the early stages.

*Mental symptoms* —The principal mental symptoms as described in the history sheets have been analysed in Table V —

TABLE V

1	Incoherence of language	280	13	Destructive	125
2	Uncommunicative	35	14	Homicidal	100
3	Complete silence	50	15	Suicidal	60
4	Talkative	45	16	Laughter	60
5	Muttering delirium	60	17	Dancing	10
6	Abusive and obscene language	120	18	Obscene postures	5
7	Excited	20	19	Sad, crying or mourning	90
8	Noisy	120	20	Dirty or filthy habits	284
9	Restless	120	21	Indecent behaviour	105
10	Sleepless	255	22	Delusions	100
11	Exaltation	35	23	Childish behaviour	20
12	Quarrelsome	65			

These symptoms changed very rapidly on admission to the hospital and in quite a large number of cases disappeared completely within a few weeks of admission. Twenty-five per cent of cases, however, showed little or no change in the beginning. In 15.0 per cent of cases there were relapses, with occasional outbursts of violence. On the other hand quite a number who were violent and abusive at first became melancholic or quiet and in very rare instances drifted to a state of dementia.

#### *Duration of insanity*

The information regarding the duration of the period of insanity was either unreliable or not available at all in all the cases, particularly for the period they were at large. It is therefore only the duration of the stay in a mental hospital



that we have analysed The duration of insanity before admission when available is included in Table VI —

TABLE VI

*Showing duration of insanity before admission into a mental hospital in 60 cases of hemp drug insanity along with their ages*

Age in years	DURATION OF INSANITY							
	1 month and under	1 to 3 months	Under 5 to 6 months	6 to 12 months	12 to 18 months	1½ to 3 years	3 to 5 years	12 years
15-20	6	4						
21-31	5	6	8	6	1			
31-40	1	3	3	1	2			
41-50	2	1	2	2	1			
51-60	1	1		2		1		
61 and up								1
TOTALS	15	15	13	11	4	1		1

A perusal of Table VI will show that 25 per cent of cases were picked up by the police and medical authorities within the first month of the beginning of the disease and another 25 per cent before the third month, there were 7 individuals who remained at large for periods of more than one year

*Duration of stay in the hospital*

The stay of 600 accepted cases of hemp drug insanity in various hospitals has been represented in Table VII This table is important from the prognostic point of view —

TABLE VII

*Showing the duration of stay in hospital in 600 cases of hemp drug insanity*

	DURATION OF STAY							
	Showing no signs on admission	1 month and under	1 to 3 months	3 to 6 months	6 to 12 months	12 to 18 months	Not cured up to 18 months	Died in the hospital
Number	60	115	55	100	65	15	155	35
Per cent	10	19 16	9 16	16 6	10 8	2 5	25 8	5 8

It would appear from Table VII that in this series, 60 persons (or 10 per cent) did not show any signs of active disease on admission, although they were admitted to the hospital after an attack of active insanity which followed the use of hemp drugs. Out of the remaining 540 cases, 115 or 19.16 per cent were cured within the first month of admission, 270 or 45.0 per cent were cured within the first six months, 335 or 55.8 per cent within the first year of admission, 350 or 58.3 per cent within 18 months and out of the remaining 190, 35 or 5.8 per cent died and 155 or 25.8 per cent were not cured and were still in the wards of mental hospitals.

The average period under treatment in various Indian hospitals varied from 3 to 8 months, and most of the patients were kept under observation before being declared as cured. It would appear that 58.3 per cent of cases recovered within 18 months which probably means that these cases are more amenable to treatment than are those of the other types of insanity. It is very difficult to express an opinion regarding the prognosis of 155 or 25.8 per cent of cases which were still detained in the various mental hospitals after a stay of 18 months. The mortality rate in this series amounted to 5.8 per cent, this, however, was not due entirely to hemp drug insanity but to intercurrent diseases.

### *Diagnosis of hemp drug insanity*

*Acute toxic hemp drug insanity*—Besides the generally accepted view that a short duration is diagnostic of hemp drug insanity, this form has points of distinction from other types of mental disorders. General care of the patient and withdrawal of the drug leads to rapid recovery\*. In regard to the different diagnostic symptoms, delirium frequently occurs. Acute mental derangements due to hemp drugs are marked by extreme vehemence of the mania. Mental, moral and muscular manifestations are more pronounced, and differ from those seen in case of alcohol and other drugs. The individual looks confused and excited, has bright shining eyes which are almost always heavily congested. He shouts, vociferates, sighs, walks quickly up and down or round his cell, and shakes the door out of its fastenings. If at liberty, he is violent and aggressive and may run amuck. These symptoms are not so pronounced in other forms of mania. Instances are on record where the patient under excitement got hold of a weapon and committed murder without any reason or provocation. The attack, however, was usually of short duration, being limited to a few days. Later, in most cases there was perfect recovery, but the patient was quite oblivious of things which took place during the period of intoxication.

*Chronic toxic hemp insanity*—The insanity produced by a long-continued and excessive indulgence in ganja or charas has also certain definite symptoms and points of its own. The patients are hilarious and full of a sense of well-being, they are as a rule good natured and trustworthy, and recover in a large proportion of cases when admitted to a hospital and after the drugs have been withdrawn.

Relapses, however, are quite common in cases of chronic form of the disease.

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\* Sudden withdrawal of the drug, unlike opium and morphine gives rise to no abstinence symptoms.

## CRIME AND HEMP DRUG HABIT.

As regards the relationship between hemp drug addiction and crime, there are instances where the addicts committed criminal acts under the effects of these drugs, especially after smoking ganja or charas, under grave provocation or in cold blood and with pre-meditation. Such instances do not necessarily prove any definite relationship between the use of hemp drugs and crime. Indulgence in alcohol undoubtedly gives rise to a feeling of bravado and courage by depressing the higher controlling cerebral centres, and there are many instances in which it has led to crimes of a very grave nature. So far as hemp drugs are concerned, however, the situation has to be viewed from a different angle. Hemp drugs are cheap and are generally used by the poorer classes who belong to the lower strata of society to which most of the criminals in this country belong. This may be an explanation of the fact that proportionately more consumers of hemp drugs, especially ganja and charas smokers, are found among bad characters than among the normal population in general.

It should also be remembered that the habitual use of these drugs impoverishes the addicts whose income is generally small. They spend a large portion of their earning on them and in consequence they have very little money left to obtain the daily necessities of life. This may lead them to commit thefts and other crimes of a similar nature. This, however, does not mean that the use of hemp drugs is entirely responsible for their misbehaviour.

So far as pre-meditated crime is concerned, particularly that of a violent nature, the rôle of hemp drugs is quite distinctive. In some cases they not only do not lead to it but actually act as deterrents. We have already remarked that one of the important actions of these drugs is to quieten and stupefy the individual so that there is no tendency to violence, as is not infrequently met with in cases of alcoholic intoxication. The result of continued and excessive use of these drugs in our experience is to make the individual timid rather than to lead him to commit crimes of a violent nature. Our opinion in this respect based on the study of a large series of addicts, is that the tendency of the drugs appears to be to develop or bring into evidence the natural disposition of the consumer and to emphasize his true character and peculiarities. For example, if he is inclined to be lazy and easy going, he will be quiet and restful, but if he is irritable or excitable he may, if interfered with, become violent. The results of the statements of the addicts in this series who volunteered information on this point are summarized in Table VIII.

From Table VIII it is clear that there were 102 or 8.24 per cent of individuals who gave the history of being convicted once and 98 or 7.92 per cent who were convicted more than once. The figures for conviction in this series are higher than those usually met with among the general population. It will also be observed that the number of those who had convictions in this series is much larger in the case of ganja and charas users than in the case of bhang. The reason no doubt is that the effects produced by smoking are rapid and more intense than when the drugs are taken by the mouth. Besides, ganja and charas are used more by the lower strata of society, which include a higher proportion of habitual criminals.

TABLE VIII

*Showing relation between addiction to hemp drugs and crime in 1,238 hemp drug addicts*

(Percentages have been worked in row totals)

Drugs	No conviction	One conviction only	More than one conviction	Totals
Bhang	704 (91.19 per cent)	48 (6.22 per cent)	20 (2.59 per cent)	772
Ganja and charas	334 (71.67 per cent)	54 (11.59 per cent)	78 (16.74 per cent)	466
Totals	1,038 (83.84 per cent)	102 (8.24 per cent)	98 (7.92 per cent)	1,238

#### USE OF HEMP DRUGS FOR HOMICIDAL AND SUICIDAL PURPOSES

It has already been remarked that hemp drugs have been used by criminals for two purposes. Firstly, to fortify themselves to commit pre-meditated crimes, and, secondly, to enable them to endure unusual fatigue or exposure to inclement weather and to stimulate them for extra exertion. In both cases the drugs are used by those who have been indulging in them habitually.

Hemp drugs have not been used for suicidal or homicidal purposes in the same way as opium. It has been stated that sometimes they are used for stupefying people with the object of robbing them. It is doubtful if stupefaction can be effected with these drugs except in those who are not accustomed to take them habitually, and very often 'dhatura' has to be mixed to obtain the desired effects.

Another difficulty in the way of using these drugs, especially ganja and charas, is that they can readily be detected by their characteristic and penetrating smell and therefore it is difficult to mix them with tobacco or other substances for the purpose of smoking without detection. It is, however, possible to utilize them for stupefying individuals who are addicted to their use or otherwise by secretly mixing with more potent drugs such as 'dhatura'. Cases have been reported where prostitutes were stupefied in this manner and robbed of their ornaments. Children are sometimes decoyed and offered sweetmeats containing hemp drugs to make them insensible and to rob them of their ornaments. Generally speaking, because of the difficulty of their administration without detection and their uncertain action, these drugs are seldom resorted to for such purposes.

#### SUMMARY AND DISCUSSION

Excessive indulgence in narcotics such as Indian hemp is apt to produce in healthy individuals and in susceptible individuals mental confusion which may lead

to delusions with disorientation and disordered movements. The use of hemp drugs if pushed further than the stage of light depression of higher centres may produce confusion of mind and restlessness. Intellectual impairment as well as disorientation may show itself in various ways, such as weakening of moral sense, habit of telling lies, prostitution, theft etc. The addict may become egotistic and unreliable and may have recourse to theft, pilfering and unnatural sex perversions. Sometimes it may release subconscious impulses and lead to violent crime.

Indulgence in Indian hemp drugs often results in illusions, delusions and hallucinations. In young individuals hallucinations of pleasant and sexual nature some time form the chief attraction for the use of these drugs. These hallucinations depend upon the personality and subconscious trend of the habitué's mind. A person with a religious trend of mind may imagine himself to be a messiah, a messenger of God or a prophet, while a person with loose morals may imagine himself in the lap of his beloved. The hallucinations and delusions in the case of Indian hemp drug are largely visual and auditory. They are mostly transitory, but in more susceptible individuals may assume a chronic form resembling paraphrenia. Delusions of a persecutory nature and sexual infidelity are dangerous and frequently lead to homicidal crimes, the power of discrimination being lost through a lack of control over the higher centres. Inquiries in various jails and mental centres revealed that in quite a number of cases, simple indulgence even in a single ganja or charas smoke was responsible for a heinous crime.

Another important point to be considered is the difference in susceptibility to the effect of hemp drugs in different individuals. Differences in effects produced in the same individual at different times and under different environments have already been recorded (Chopra and Chopra, *loc cit*), but it may be noted that individuals may react differently after head injuries or following special nervous and mental strain.

It is well known that the cerebral centres (higher centres) are responsible for keeping in check the lower centres upon which the behaviour of an individual depends. Hemp drugs and other narcotics depress the higher centres to begin with. Moderate interference with these may not be dangerous and sometimes may even be helpful, as for instance in removing inferiority complexes and temperamental difficulties. With frequent repetitions of the dose, however, harm is likely to result through prolonged inhibition of the higher faculties of the mind, and this may lead to permanent changes in character and even the production of such conditions as megalomania, irritability of temper, boastful nature, etc. Fits of aggressive mania are not infrequently observed after indulgence in hemp drugs. Impulses of a suicidal and morbid nature have been known to occur with drugs such as bhang when taken in large doses, especially during a period of mental depression. The higher centres when kept under constant repression undoubtedly suffer, they are the last to develop and first to succumb to the action of these drugs. The lower centres also suffer through over-action, but they are more resistant and do not show signs of degeneration till late. Our studies in mental hospitals, in the field (*vide* Tables I and VII) and in prisons show that not infrequently addiction to hemp drugs was the immediate cause of a sudden unbalance of mind and the commission of a crime.

Deliberate indulgence in hemp drugs by religious mendicants to induce a state of frenzy for impressing the onlookers with their supernatural powers is frequently found. Frenzy is also deliberately induced to enable the subject to carry out enterprises of a difficult and dangerous nature, either planned beforehand or only decided upon on the spur of the moment

A milder condition is sometimes induced by criminals to enable them to keep up their courage while committing offences. Though they differ in degree yet the two uses are essentially similar. In the first instance it may be followed by murder, while in the second by robbery, theft and the like. The striking example of the abuse of these drugs occurred in the sect Hassassins in Persia who committed terrible deeds under the influence of hemp drugs. The frenzied state is more likely to be induced by a cumulative effect of repeated doses of hemp drugs than by a single dose.

#### ACKNOWLEDGMENT

We are very grateful to Lieut-Colonel J E Dhunjibhoy, I M S, for the facilities he has given us for these studies and for his advice at any stage of this work.

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## THE EFFECT OF TRACHEAL STENOSIS ON THE LUNG OF THE RAT

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[Received for publication, September 22, 1941]

THE following experiments were devised to study the relationship between the size of the stenosis of the trachea and the intrabronchial pressure, and the effect of the size of the stenosis on the lung peripheral to the stenosis —

### METHOD

*Calibration of the stenoses* —An ordinary glass stop-cock with a glass-rod sealed to its handle was fixed in position on a board. Using the glass-rod as a pointer, three arbitrary degrees of closure of the stop-cock were marked so that they could be repeated accurately at will. The bore of the stop-cock was 2 mm in diameter.

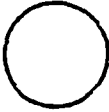



The period of time required to pass 100 c c of kerosene under a constant pressure of 80 mm of Hg through each degree of stenosis could then be compared with the times determined on the known degrees of stenosis in the stop-cock. In Table I the time required and approximate appearance of each degree of stenosis of the stop-cock are given. When the trachea and major bronchi to the hila of the lungs are included in the system, with the stop-cock open, the kerosene time was between 12 and 13 seconds. If only the trachea was included, the time was between 10 and 11 seconds. In the animals in which stenoses had been produced surgically, at the end of the experiment, the trachea was removed and tied over the cannula. Its kerosene time was then determined. Obviously the effect of a stenosis on the kerosene time will be much greater than on air since its viscosity is so much greater.

*Production of stenoses* —The tracings presented were taken on rats in which stenoses had been produced in one of three manners. Method 1 consisted in the use of the calibrated stop-cock connected directly to the trachea of a normal rat by a cannula.



TABLE I

*Showing time required and approximate appearance of each degree of stenosis*

Degree of stenosis	Diagram 5 ✓	Kerosene pressure, mg Hg	Time in seconds per 100 c c
0		80	8
1		80	9
2		80	18
3		80	35

Method 2 The trachea of the rat was exposed after the animal had been anæsthetized with ether. A wire 1 mm in diameter was placed next to the trachea. A silk tie was tied tightly around both the wire and the trachea, and the wire removed immediately, thus leaving an opening in the trachea approximately 1 mm in diameter. The tracings were taken on the animals which survived this procedure by fourteen days.

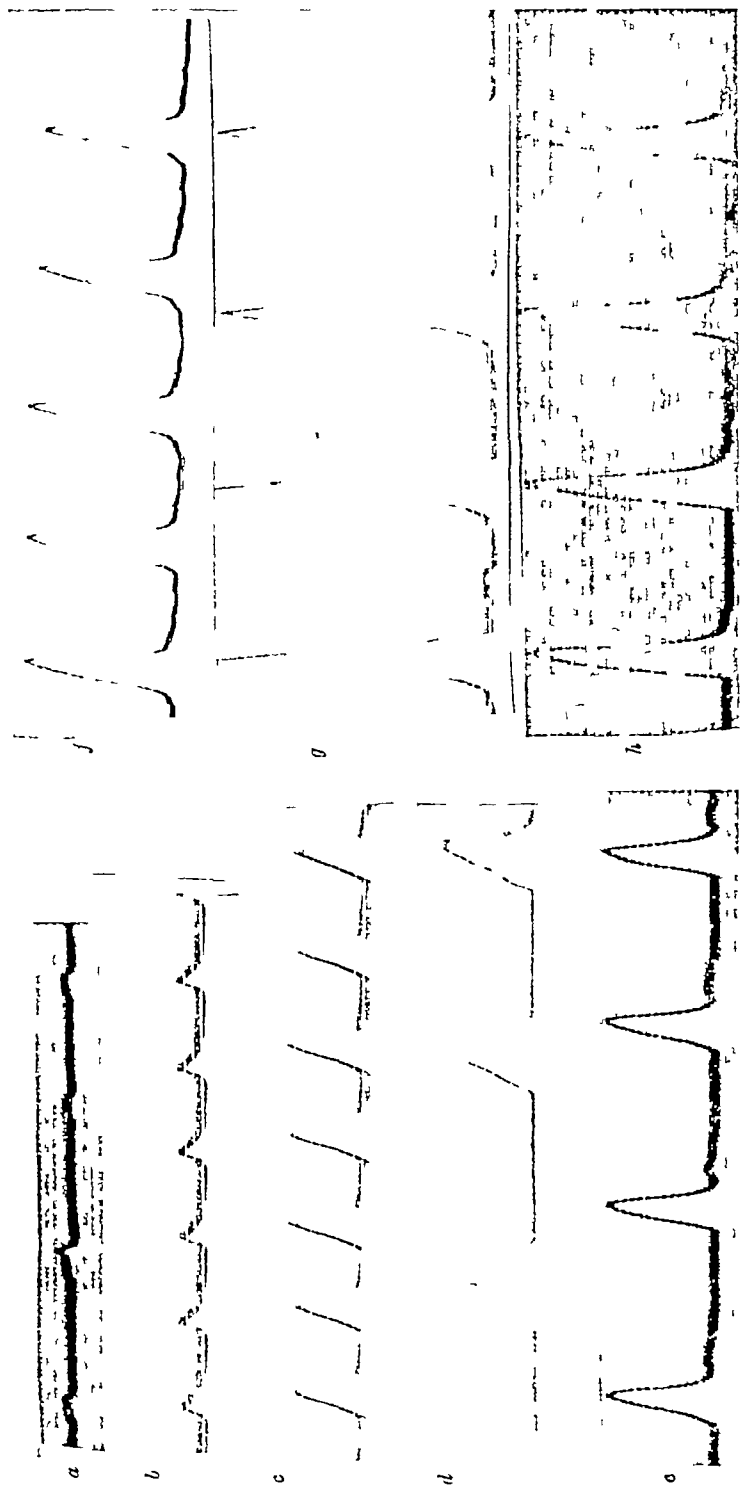


Fig 1

Tracing	Experiment	Degree of stenosis	Pressure change	Tracing	Experiment	Degree of stenosis	Pressure change
a	Normal	2	+0, -1 cm	e	Pneumonectomy	3	+0, -7 cm
b	Normal	2	+0, -3 "	f	Pneumonectomy	3	+0, -7 "
c	Normal	3	+0, -5 "	g	Normal	4	+0, -10 "
d	Normal	3	+0, -5 "	h	Pneumonectomy	4	+0, -10 "

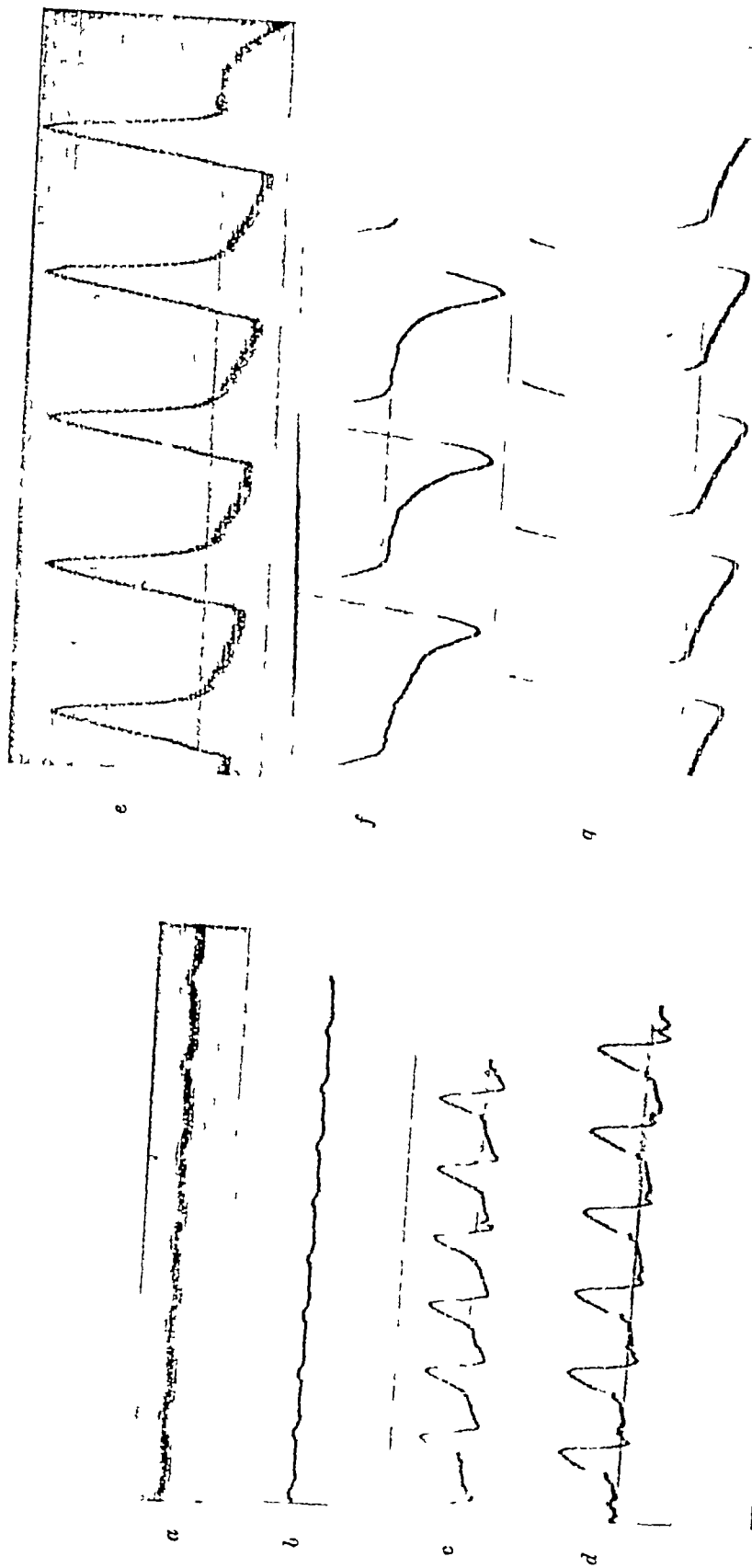


Fig. 2

Tracing	Experiment	Degree of stenosis	Pressure change	Tracing	Experiment	Degree of stenosis	Pressure change
a	Phrenicectomy	2	$\pm 0.5$ cm	e	Phrenicectomy	1	$+2$ , $-8$ cm
b	Phrenicectomy	2	$\pm 0.5$ "	f	Phrenicectomy (acute)	4	$+5$ , $-5$
c	Phrenicectomy	3	$\pm 1.5$ , $-4$ "	g	Phrenicectomy	1	$+3$ , $-9$
d	Phrenicectomy	3	$+1$ , $-4$ "				

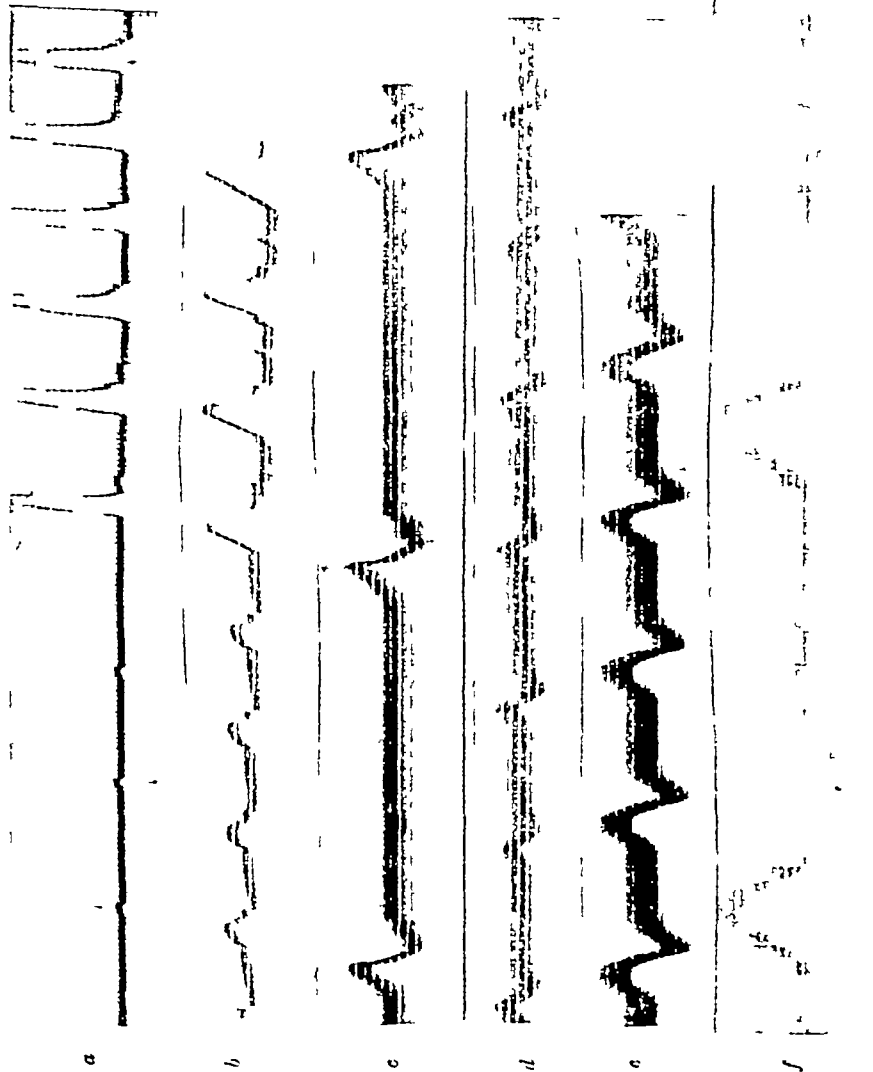


FIG. 3

Tracing	Experiment	Degree of stenosis	Pressure change	Tracing	Experiment	Degree of stenosis	Pressure change
a	Normal	From 1 to 4	+0, -05	d	Stenosis 27 days	Between 1 and 2	+1, -15
b	Normal	2 to 3	+0, -2	e	Stenosis 32 "	" 1 and 2	+2, -3
c	Stenosis 32 days	Between 1 and 2	+2, -3	f	Stenosis 35 "	" 2 and 3	+3, -55

**Method 3** The trachea of a young rat was exposed and a silk tie placed snugly about it without encroaching on the lumen. As the animal grew, a stenosis was gradually produced. These animals were allowed to survive a longer period of time.

Similar stenoses were produced in animals in whom phrenicectomy and pneumonectomy had been performed after the manner previously described (method 1).

*Method of recording*—The tracings were made after the animals had been given 0.1 c.c. of nembutal (1 g. per c.c.) intraperitoneally. The trachea was then exposed and one opening of a T-shaped cannula inserted in the trachea below the stenosis. To the second limb of the T the bit of trachea including the stenosis was tied. The third limb of the T was connected to a Frank capsule. The light beam was then recorded on a moving film. Following each tracing a water manometer was inserted in series with the capsule so that the pressure changes represented by the deflection of the light beam were determined.

*Results*—Typical tracings are illustrated in Figs. 1, 2 and 3. The condition of the experiment, the degree of stenosis and the pressure change are given for each tracing.

After a satisfactory record had been made, the abdomen was opened, the aorta cut, and the heart and lungs trimmed, blotted and weighed. The weights were then compared to normals for this strain of animals (1, 2). No animal in which any pulmonary disease was present was used. The percentage change of the weights of the hearts and lungs of these animals is given in Table II.—

TABLE II

*Effect of tracheal stenosis on the weight of the heart and lung*

Number of rats	Duration, days	Average body weight, g	Average heart weight, mg	Percentage increase heart weight, mg	Average total lung weight mg	Percentage increase lung weight, mg
8	11	148	510	0	948	18
12	35	117	507	18	861	24

When the organ-weights in the individual animals are compared to the degree of stenosis, it is seen that the greater the stenosis and the longer the time it has been present, the greater is the increase in the organ-weight over the normal (Table III).

TABLE III

*The relationship of organ-weight to the degree of stenosis*

Body weight, g	Duration of stenosis, days	Heart-weight, mg	Per centage change	Lung weight mg	Per centage change	Kerosene time	Approximate size of stenosis	Condition of animal
							Between	
64	32	390	28	630	30	21	2-3	Thin
130	32	590	19	1 100	35	26	2-3	,
84	32	350	0	590	11	11	1-2	„
105	14	467	10	674	12	12	1-2	Normal
82	14	320	13	518	8	16	1-2	„
76	35	372	15	1,220	66	37	3	Thin
98	14	450	16	620	5	9	0-1	Normal
100	27	530	25	770	25	20	1-2	„
122	30	450	0	700	3	11	1-2	„
122	13	480	7	870	20	32	1-2	„

It should be noted that in calculating the percentage change in organ-weight, individual animals are compared to averages for normals

## DISCUSSION

The tracings of the respiratory effort of normal animals in the presence of acute tracheal obstruction uniformly show an inspiratory effort only. No positive pressure is developed. This is true even when half the pulmonary tissue is removed (Fig 1-e). When, however, the diaphragm is paralysed following phrenicectomy, a positive pressure is developed (Fig 2). This is due in part to the fact that the diaphragmatic tone of the normal diaphragm no longer resists the upward thrust of the abdominal viscera with the contraction of the abdominal muscles. The lung collapses to a greater extent, thus developing a positive pressure on expiration. Inspiration is not as great as in the normal animal, and to maintain respiration, expiration must be greater. A reading taken immediately following phrenic avulsion in one instance (Fig 2-f) showed a pressure reading of minus 5 cm. of water on inspiration to plus 5 cm. of water on expiration.

After a stenosis has been present for some time, tracings show that again a positive pressure is developed on expiration. The respiratory effort becomes more efficient in the presence of a stenosis by becoming deeper as well as slower (Fig 3).

The presence of the tracheal stenosis increased the mean thoracic volume. Since it has been demonstrated that stretching is the adequate stimulus for growth of the lung (1, 2), it could be expected that the lung-weight would increase as it did (Table II). The heart-weight increased because of the work required in breathing. The figures for this increase are probably high as the animals did not eat well and are somewhat underweight when compared to normals of the same age.

#### CONCLUSIONS

Apparently, in the rat, obstruction to the bronchial tree of slight degree is not significant. When the obstruction does become significant, compensation takes place by a slowing and deepening of the respiratory rate as well as an increase in the mean respiratory volume. Following the increase in mean thoracic volume, because of the stretch on the lung, an increase in the amount of pulmonary tissue takes place.

## NOTICE.

### Economy in the Use of Ipecacuanha and Emetine

AMEBIC DYSENTERY is common in India and accounts for a fair amount of mortality and morbidity, especially amongst the members of the European communities resident in the tropics. In the treatment of this condition, Emetine, one of the principal alkaloids of Ipecacuanha, is considered a specific and is very largely used.

Ipecacuanha is not indigenous to India. Attempts have been made to grow this plant in suitable localities in India but the indigenous supply has never been adequate to meet the local demands. India, therefore, remains largely dependent on foreign sources of supply.

Owing to conditions produced by the war supplies of this essential drug have been scarce and irregular. In the interests of the sick it is desirable that a general effort on the part of medical men should be made to conserve stocks of this important remedy, so that those who really need it will not be deprived of its valuable curative properties.

The extent to which Ipecacuanha enters into popular therapeutics is surprisingly large. Much of this is in the form of *Pulvis Ipecacuanhæ et Opii* (Dover's Powder), which is almost an everyday prescription in influenza, chill, coryza and slight cough. It is often used to 'abort' an attack of cold and also to check mild diarrhoea. Its diaphoretic and astringent actions are mainly responsible for its popularity. Therapeutically there appears to be no particular advantage in using *Pulvis Ipecacuanhæ et Opium* to bring about these effects. It is the Opium in the preparation, and not the Ipecacuanha, which brings about the diaphoretic and astringent actions. The astringent action can be very easily obtained, probably more conveniently, by the use of *Pulvis Cretæ Aromaticus cum Opio*. For diaphoresis practitioners have at their disposal a large selection of coal-tar remedies including salicylates and their derivatives. The use of Dover's Powder can, therefore, be conveniently reduced by encouraging the use of various substitutes which may bring about similar clinical results.

A significant contribution towards economy in the use of Ipecacuanha may also be made by exercising a little more judiciousness and care in prescribing the galenicals of Ipecacuanha, of which *Vinum Ipecacuanhæ* (now *Tinctura Ipecacuanhæ*) is the most widely employed. *Tinctura Ipecacuanhæ* is a common ingredient of 'Sedative Expectorant' nuxtures frequently seen in the Pharmacopœias of various hospitals and out-patients' clinics. *Tinctura Ipecacuanhæ* is neither a very rapidly acting nor always a trustworthy expectorant. In many types of



cases of bronchitis, the use of such mixtures is not of any particular advantage and *Tinctura Ipecacuanhæ* may be replaced by *Vinum Antimoniale* or *Tinctura Scillæ*. Only in pediatric practice is there some justification for its continued use in war time.

*Pulvis Ipecacuanhæ* is gradually going out of fashion but prescriptions are still seen where this has been used either for its emetic or for its cholagogue action. Such uses are a relic of the old days and cannot be supported by rational therapeutics. Ipecacuanha has now been shown to be a poor emetic and its supposed cholagogue action is also doubted by modern pharmacological investigators. Its use in such conditions therefore need no longer be advocated.

Whatever may have been the justification of such haphazard use of Ipecacuanha in pre-war days, it is the clear duty of all medical men to stop such practice in these abnormal times when there is a shortage of this essential remedy. If every medical man employs the same rigid criterion in the use of Ipecacuanha as they do in the diagnosis of their cases, much waste in the use of the preparations and galenicals of Ipecacuanha could be avoided and stocks of Ipecacuanha could be reserved for the extraction of Emetine, whose value in the treatment of amœbiasis has been fully established.

A F MACCULLOCH,

NEW DELHI,  
6th December, 1941

Secretary, Medical Stores Supply Committee  
(Office of the Director-General, Indian Medical Service)

ACTION OF *V CHOLERÆ* AND EL TOR TYPE  
STRAINS ON GOAT'S RED  
CORPUSCLES

BY

W D B READ,

S R PANDIT,

AND

P C DAS

*(From the Cholera Inquiry under the Indian Research Fund Association,  
All-India Institute of Hygiene and Public Health, Calcutta )*

[Received for publication November 10, 1941 ]

SINCE Greig (1914) published his account of findings on the power of plain broth cultures of large numbers of vibrios isolated in Calcutta to attack goat's red cells, it has been tacitly accepted by most workers that the classical case vibrio, *V cholerae*, has no hæmolytic power against the red cells of that animal. In view, however, of the findings of de Moor (1938) in the Celebes Islands of hæmolytic agglutinable vibrios in connection with cases indistinguishable clinically and epidemiologically from cholera and their surroundings, the validity of the Greig test as a basis of distinction has been questioned with considerable reserve by certain of the Dutch workers, notably Otten (1939). de Moor (1939), however, now appears to support the validity of the test.

Read and Pandit (1941) show that there is an almost complete correlation between the Indian sources from which the agglutinable vibrio can be isolated and the Greig test, slightly modified. This is, however, out of line with the findings in the Celebes Islands.

This communication gives the results of submitting strains of fairly recent origin from Indian and Celebes sources together with a few classical El Tor strains for comparison to tests on goat's blood cells carried out under different conditions of temperature and oxygen supply both on solid and in fluid media.

The strains used are given in Table I —

TABLE I  
*Last of strains employed*

Strain number	Source	Place of isolation	Date of isolation	Serological type
TMCH 1800 1	Case	Calcutta	26-5-36	Inaba
CST 100		,	2-10-40	Rough
CST 101		,	2-10-40	Ogawa
CST 202	,	,	8-12-40	Intermediate
CST 203	,	,	8-12-40	Ogawa
CST 204			8-12-40	,
CST 205		,	8-12-40	,
CST 206	"	,	8-12-40	"
CST 207	,		8-12-40	"
CST 208	"	,	8-12-40	"
BST 02	,	Khulna district Bengal	3-7-39	"
BST 1470 1	"		21-2-40	
BST 1738 1		,	27-3-40	
MST 2817 1		,	6-3-40	Inaba
MST 3077	,		28-1-40	"
El Tor No 1863 (Damang)	"	Celebes Islands	9-3-38	Intermediate
El Tor No 757 (Rahim)	"	"	22-9-37	Ogawa
El Tor No 7579	"	"	2-7-40	Intermediate
El Tor No 301	"	"	18-1-40	"
El Tor No 567	"	"	7-11-39	"
El Tor No 7617	"	"	3-7-40	"
El Tor No 7632	"	,	3-7-40	,
BST 1446 1	Contact stool	Khulna district, Bengal	16-2-40	Ogawa
MST 2775 1	"	"	4-3-40	Inaba

TABLE I—*contd*

Strain number	Source	Place of isolation	Date of isolation	Serological type
VST 3380 1	Contact stool	Khulna district, Bengal	23-4-40	Ogawa
MST 2831 1	"	"	6-3-40	Inaba
El Tor No 7804	"	Celebes Islands	15-7-40	Intermediate
El Tor No 681	"	"	2-12-39	"
El Tor No 7951	"	"	17-7-40	"
El Tor No D 13	Old El Tor strain	"	"	"
El Tor D (N C No 3662)	"	"	"	Ogawa
El Tor D (number lost)	"	"	"	"
El Tor No D 1408 (N C No 1548)	"	"	"	"
El Tor (N C No 3657)	"	"	"	Inaba
El Tor No 1905 (Gotschlich)	"	"	"	"
BW 44	Contact water	Khulna district, Bengal	3-7-39	Ogawa
BW 90	"	"	13-7-39	"
BW 793 1	"	"	16-2-40	"
VW 990 1	"	"	23-12-39	Inaba
BW 996 1	"	"	10-4-40	Ogawa
MW 1416 1	"	"	4-3-40	Inaba
El Tor No 7729	"	Celebes Islands	9-3-40	Intermediate
El Tor No 103	"	"	15-11-39	"
El Tor No 7585	"	"	2-7-40	"
El Tor No 7587	"	"	2-7-40	"
El Tor No 7533	"	"	27-1-40	"
El Tor No 970	"	"	12-1-40	"
BW 92	Non contact water	Khulna district, Bengal	13-7-39	Ogawa.
MW 120T	"	"	17-7-39	"

TABLE I—concl'd

Strain number	Source	Place of isolation	Date of isolation	Serological type
BW 495	Non contact water	Khulna district, Bengal	30-11-39	Inaba
MW 160		"	24-7-39	Ogawa
MW 136		"	19-7-39	"
BW 203		"	17-8-39	"
MW 142		"	19-7-39	"
MW 275	"	"	21-8-39	"
MW 893	"	"	11-12-39	Inaba
MW 1169		"	25-1-40	Ogawa
MW 2019		"	12-7-40	Inaba
SW 73	"	Tatta, Sind Province	24-10-40	Intermediate
SW 351		"	27-10-40	"
PW 3551	"	Patna district, Bihar Province	11-8-40	"
PW 4332	"	"	20-8-40	"

Inaba Serological type 'O' group I, Gardner and Venkatraman (1935) sub type Inaba

Ogawa Serological type 'O' group I, Gardner and Venkatraman (*loc cit*) sub type Ogawa

Intermediate Reacting with type specific serum of both sub types Inaba and Ogawa

Rough Serological rough type of 'O' group I, Gardner and Venkatraman (*loc cit*), Yang and White (1934)

Celebes strains were received through the courtesy of Dr de Moor. Old El Tor strains, mostly through the courtesy of Major C L Pasricha, I M S, from the School of Tropical Medicine, Calcutta

The exact history of all Celebes Islands strains is not certain

The tests to which these vibrios were submitted were as follows —

1 *Modified Greig test* —The original tests as done by Greig (*loc cit*) were carried out in an untrypsinized alkaline broth. In this series Douglas broth (Douglas, 1914) was prepared with 0.85 per cent NaCl, as it was found in certain instances that controls would hæmolyse when 0.5 per cent NaCl was employed.  $4 \times \frac{3}{8}$  test-tubes were employed, the total volume of fluid in each tube being 1.5 c.c.

Strains were kept on agar slopes and sub-cultured every 14 days. In other respects the technique recommended by Greig was adopted.

The results of tests were as given in Table II —

TABLE II  
*Results of modified Greig test*

Source	Place of isolation	Number of strains showing hæmolyasis	Number of strains not showing hæmolyasis
Case	Calcutta	0	15
	Khulna		
	Celebes Islands	7	0
Contact stools	Khulna	0	4
	Celebes Islands	3	0
Old El Tor strains	?	5	1
Contact water	Khulna	1	5
	Celebes Islands	6	0
Non contact water	Khulna	14	1
	Bihar		
	Sind		
TOTAL		36	26

In the above tests it will be seen that there was a very high degree of correlation between the sources of Indian strains and the results of the test, Celebes Island strains were universally hæmolytic but it has not been possible to verify their epidemiological history, though they were almost certainly isolated from sources in contact with the disease. One old El Tor strain (obtained through the School of Tropical Medicine, Calcutta) proved non-hæmolytic.

In further studies strains will be classified as Greig-positive and Greig-negative strains.

*Effect of temperature at which the test is set*—In the Greig test after a preliminary incubation for two hours at 37°C the test is set in the ice-box. The effect of increasing the temperature at which this test is set to 37°C was tested both in broth cultures and on blood-agar plates. Cultures in Douglas broth with 0.85 per cent NaCl were grown for 1, 2, 3, 5, 10 and 15 days and the goat's cells then added and in addition blood-broth tubes containing washed cells at 2.5 per cent concentration were inoculated and incubated. In blood-broth tubes, 1.5 c.c. total fluid was employed. The red cells were added

to the broth and the culture inoculated before the cells had deposited. Blood agar was poured in uniform-sized plates with a fixed quantity of 2 per cent Douglas-broth agar and 10 per cent of washed red cells, so that the depth of the medium was 4 mm. One organism only was grown on each plate.

TABLE III

*Results of tests on cultures in fluid media grown at 37°C aerobically and test set at 37°C aerobically*

Hours at which reading was taken —	24						48						72					
Age of cultures in days —	1	2	3	5	10	15	1	2	3	5	10	15	1	2	3	5	10	15
<i>Greig positive organisms</i>																		
Number of strains showing hæmolysis —																		
Complete	35	36	25	6	3	2	36	36	31	6	4	2	36	36	32	11	5	2
Partial	1		5	2	2	4			5	7	3	6			4	22	2	10
Trace			6	4	3	5				7	7	7				3	15	12
Nil				24	28	25				16	22	21					14	12
<i>Greig negative organisms</i>																		
Complete																	2	
Partial							2		12	12	10	9	12	17	16	19	13	16
Trace				2	3	1	3	8	7	4	7	4	9	7	5	3	8	3
Nil	26	26	26	24	23	25	21	18	7	10	9	13	5	2	5	2	5	7

In all these experiments complete hæmolysis was recorded when all the red cells that had sedimented to the bottom of the tube had disappeared and debris only remained or when the condition was such that it was difficult to decide whether or not the red cells had been removed. No notice was taken of the colour of the supernatant or of the distribution of the lysed hæmoglobin in it. The red cells once they had sedimented were not disturbed. Trace hæmolysis when produced in unrestricted oxygen supply was always the so called 'cap hæmolysis' and could not be estimated in this way, but was read as the smallest change that could be detected on observation, the criterion being a small layer of clear purple fluid superimposed on sedimented red cells. Partial hæmolysis represented an intermediate stage between 'complete' and 'trace' and was always accompanied by the presence of sedimented red cells. A recording of complete or trace hæmolysis therefore gives some indication of the comparative hæmolytic power of different strains for a given concentration of red cells used, but this is less the case when a recording of partial hæmolysis is made. This system was, however, found largely to obviate variation in readings from alterations in the colour of the supernatant fluid, which in later readings may occur to a marked degree.

From Table III it will be seen that a large number of Greig-positive organisms fail to show hæmolysis after three days. There is some diminution of activity in three-day cultures. Late readings of the older cultures show an increase in the number of partial reactions. Early cultures give almost universally complete reactions. Two strains differed from the rest in retaining their hæmolytic activity unimpaired up to 15 days and one more up to ten days. In blood broth the Greig-positive strains showed hæmolysis after 24 hours incubation.

TABLE III-a

*Results of tests in blood broth containing 2.5 per cent washed cells incubated at 37°C*

Days at which reading was taken —	Greig positive organisms					Greig negative organisms				
	1	2	3	4	5	1	2	3	4	5
Number of strains showing hæmolysis —										
Complete		20	33	34	34					7
Partial	36	7	3	2	2		2	12	21	19
Trace							1	6	4	
All						26	23	8	1	

With 24-hour readings of Greig-negative organisms trace reactions are recorded only with occasional older cultures. From 48-hour readings of cultures of all days there are positive reactions, but more occur amongst the older cultures. There are more positive readings in 72-hour readings than in 48-hour ones. The reactions are almost all partial in type. In blood broth the Greig-negative strains show hæmolysis from 48 hours' incubation onwards, increasing in number and degree up to five days. A considerable degree of browning of the hæmoglobin occurs in tubes that are incubated beyond 72 hours.

It will be noticed that the increase in the number of the partial and trace hæmolytic reactions obtained in the late readings of the older cultures of Greig-positive strains is suggestive of the type of reactions obtained with the Greig-negative strains.

With regard to repeat tests with any given strain it can be said that minor variations occurred only with young cultures but that more variations occurred with older cultures, especially with late readings. 24-hour readings were almost universally stable from test to test. Thus the Greig test is supported by early readings when the test is set at 37°C in fluid medium but not by late readings. The late readings differ in their degree from the early readings. In no case is in



early reaction of the complete type produced by prolonging the incubation of the cultures, indeed the effect of such prolongation on most of the Greig-positive strains is to decrease the early activity

*Action on blood agar*—Blood-agar plates were incubated aerobically up to five or seven days. Naked-eye observations were alone recorded. In order to study action on the lysis of the blood cells, a record was made of the following points —

- 1 Presence or absence of detectable reduced hæmoglobin below the area of growth
- 2 Degree of clearing of the blood cells subjacent to the area of the growth
- 3 Presence or absence of zone area external to the growth area in which alteration, diminution or clearing of the cells occurred
- 4 Degree of such change in the zone area
- 5 Presence or absence of visible green pigment. This was estimated by observation with oblique transmitted light, the plate being held opposite to a window shutter
- 6 Presence or absence of variant growth in the zone area

TABLE IV

*Results of tests on agar containing 10 per cent washed cells incubated at 37°C*

Days at which reading was taken —	Greig positive organisms					Greig negative organisms				
	1	2	3	4	5	1	2	3	4	5
Number of organisms showing —										
Reduced hæmoglobin under growth area —										
Present	28	9	3	2	2	4	12	18	11	8
Absent	8	27	33	34	34	22	14	8	13	16
Medium subjacent to growth area —										
Blackened	2	0	0			22	13	7	2	1
Blood cells partially cleared	15	5	2			4	17	12	16	5
Blood cells completely cleared	19	31	34	36	36		2	7	16	16

TABLE IV—*conold*

Days at which reading was taken —	<i>Greig positive organisms</i>					<i>Greig negative organisms</i>				
	1	2	3	4	5	1	2	3	4	5
Medium in zone area —										
Size of zone in mm	$\frac{1}{2}$ -3	$\frac{1}{2}$ -7	2-14	4-17	7-17		$\frac{1}{2}$ -3	$\frac{1}{2}$ -4	$\frac{1}{2}$ -7	1-10
Blackened	2									2
Blood cells partially cleared	27	10	2	2	2		1	3	2	4
Blood cells completely cleared.	7	26	34	34	34		2	5	14	17
No zone						26	23	18	9	3
A B—Strains which showed no zone formation in five days in all except 2 cases showed a fully or partially cleared zone in seven days										
Variant growth —										
Number of strains in which—										
Present	0	10	20	21	21	0	1	1	1	1
Absent	36	26	16	15	15	26	25	25	25	25
Pigment —										
Number of strains—										
Producing	1	27	33	33	33	0	2	7	16	18
Not producing	35	9	3	3	3	26	24	19	10	8

Viewed on the basis of the above criteria the reaction in both Greig-positive and Greig-negative organisms seems to follow a similar series of changes, but the rate at which the changes occur varies. The first change is a blackening of the growth area (as recorded by transmitted light). This is not entirely a question of the opacity of the growth because it has no relation to the thickness of the growth and can be detected as an early sign of zone formation external to the area of growth. In the case of Greig-positive organisms the rate of change is so rapid that this appearance is rarely present in one-day cultures though it is by far the

commonest appearance in Greig-negative organisms. The next change is a partial clearing of the red cells subjacent to the growth area leaving an area where reduced hæmoglobin is easily visible. This area is naturally more evident when the growth opacity is small. In the case of the Greig-positive organisms this area is visible in one-day cultures and has usually disappeared in two-day cultures. In the case of the Greig-negative organisms this purple area persists in many strains up to five days and its appearance and disappearance is more gradual in all. The clearing of the red cells subjacent to the growth area precedes the zone formation though both are present in Greig-positive organisms in one-day cultures. The clearing of the red cells in the zone area is at first incomplete, the clearing spreading outwards until the whole is completely cleared. As soon as it reaches the latter stage the green pigment becomes evident. It appears that those organisms in which the pigment is not detected do not completely clear the red cells. Naked-eye observation will not reveal at what stage the pigment is first produced as it may be masked by the incomplete clearing of the red cells in the early stages. With very few exceptions the final appearance in the case of all strains tested was the same if growth was sufficiently prolonged, i.e. a dark or pale growth area free of reduced hæmoglobin surrounded by a broad green zone completely or nearly completely freed of red cells. Where the zone area is rapidly formed and cleared the cultures tend to throw out protrusions of variant growth which will grow for two to three days across the zone area. The cultural appearance of this growth is completely different from the parent growth though there appears to be no other essential difference in the organisms so growing. This variant up to three or four days' incubation may cover the entire zone area. After that the zone formation outstrips it and a clear zone is formed externally. Only one Greig-negative strain which produced an early zone gave rise to this appearance though many of the Greig positives did so. Two of the Greig-positive organisms failed to destroy the reduced hæmoglobin subjacent to the growth area in five days and they also failed to clear all the red cells in their zone area. In addition pigment was not detected. There are also other points of difference between the latter cultures and the other Greig-positive organisms and they will form the subject of later comment. To summarize there is no essential difference in the growth appearance on blood agar produced by both Greig-positive and Greig-negative organisms but there is a very marked difference in the rate at which the changes are produced. The findings are analogous with those in blood broth enumerated above.

In blood-agar plates incubated anaerobically in McIntosh and Fildes' jar, the Greig-positive organisms showed an incompletely cleared zone around the growth even in 24 hours but in the Greig-negative organisms there was absolutely no zone formation around the area of growth even after nine days. There was no green pigment in either the Greig positives or negatives.

*Effect of reducing the temperature at which the test is set*—This test only differed from the Greig test in that 1-, 2-, 3-, 5-, 10- and 15-day cultures in Douglas broth grown at 37°C were used and the test set at 12°C up to three days. The preliminary incubation at 37°C for two hours was dispensed with the cultures being placed as quickly as possible in the frigidaire, usually half an hour after

the blood was added to the cultures (bench temperature about 80°F) The tests of the Greig-positive organisms are summarized in Table V —

TABLE V

*Results of tests in cultures in fluid media of Greig-positive organisms grown at 37°C aerobically and test set at 12°C aerobically*

Hours at which reading was taken —	24						48						72					
Age of cultures in days —	1	2	3	5	10	15	1	2	3	5	10	15	1	2	3	5	10	15
Number of strains showing hæmolysis —																		
Complete	33	36	13	5	1	1	33	36	13	5	1	1	33	36	13	5	1	1
Partial	3		5		2		3		6		2		3		7		2	
Trace			6						6	1					8	1	2	
Nil			12	31	33	35			11	30	33	35			8	30	31	35

*Greig-negative organisms* —These were in a parallel series to the Greig-positive organisms and completely failed to lyse the red cells

Reduction of the temperature at which the test is set has reduced the number of positive reactions from three-day cultures of Greig-positive organisms. Presumably, therefore, it will reduce the power of all strains to cause lysis though this is not evident from the results with the younger cultures. In addition the increase in the number of partial positive reactions in the later readings of the older cultures is abolished. One culture retains its hæmolytic power up to 15 days and three up to ten days. These four include the three cultures which retained their power to effect complete hæmolysis up to 15 or 10 days when the test was set at 37°C and also the two cultures which failed to form evident pigment on blood-agar plates. In parallel with the above the power of the Greig-negative organisms to effect late hæmolysis seen at 37°C was completely abolished at 12°C.

*Effect of altering the oxygen tension* —The effect of this was studied both by restricting the access of oxygen during the period of growth of the cultures and also in the case of aerobically grown cultures during the period in which the test

was set for examination. Partial restriction was secured by the use of vaseline seal and full restriction by the use of McIntosh and Fildes' jar. Temperature was maintained at 37°C.

*A Cultures grown under vaseline seal and set under vaseline seal* (broth before inoculation was boiled and quickly cooled)

TABLE VI.

*Results of tests in cultures in fluid media of Greig-positive organisms grown at 37°C under vaseline seal and test set under vaseline seal at 37°C*

Hours at which reading was taken —		24						48						72					
Age of cultures in days —		1	2	3	5	10	15	1	2	3	5	10	15	1	2	3	5	10	15
Number of strains showing hæmolysis —																			
Complete		30	35	32	8	14	21	36	35	35	30	27	31	36	36	35	33	33	33
Partial		6	1	4	28	22	15		1	1	6	9	5			1	3	3	3
Trace																			
Nil																			

*Greig-negative organisms* — Cultures of one, two and three days' growth only were tested up to 72-hour readings, all of which were negative.

With the above may be compared the results of inoculating blood broth and incubating under vaseline seal. This was repeated on several occasions and although the majority of Greig-negative strains failed to cause lysis even after nine days' incubation, certain strains would do so even after 24 hours' growth in any given series.

On successive tests, however, it was seldom the same strains that behaved in this way and the conclusion was formed that the results must be due to some difference in the way the test was set up in the case of individual tubes. Greig positives gave a partial reaction in 24 hours and a full reaction in 48 hours, this in spite of a very small increase in opacity in the cultures.

*B Cultures grown in the anaerobic jar and test set in the anaerobic jar* — A preliminary trial showed that Greig positives could not fully develop their hæmolytic power till they had been incubated three days, also that continuous

opening and closing of the jar daily would affect the results obtained. Cultures were therefore set for test after three days' growth and in the case of the Greig-negative organisms also after nine days' growth.

The results obtained were as follows —

*Greig-positive organisms* —

TABLE VII

*Results of test in cultures in fluid media grown at 37°C in McIntosh and Fildes' anaerobic jar and test set at 37°C in McIntosh and Fildes' jar*

Hours at which readings were taken —	<i>Greig positive organisms</i>		
	24	48	72
Age of cultures in days —	3	3	3
Number of strains showing hæmolysis —			
Complete	8	35	36
Partial	28	1	
Trace			
Nil			

It will be noticed that the test took longer to develop the complete reaction as compared with the test done aerobically either at 37°C or at 12°C. Similar results could be obtained if the cultures were grown anaerobically at 37°C and the test set at 12°C aerobically, in this case, however, five of the Greig positives remained negative at 24-hour readings and two at 72 hours.

*Greig-negative organisms*—No strain gave any reaction under the same conditions enumerated above.

The matter was pursued further by examining these strains in blood broth. The Greig positives reacted as before. The Greig negatives gave results which were out of line with those enumerated above, viz that a large majority of cultures gave hæmolysis of varying degree, of which 12 to 23 per cent were of the complete type. With a strictly controlled experiment it was found, however, that on opening the jar on the ninth day only three strains showed hæmolysis of the partial type but all the rest including the uninoculated blood-broth control gave a faint trace of hæmolysis. As both aerobic and anaerobic plating of the cultures showed absence of contamination in the cultures and sterility of the uninoculated control tube, the conclusion was arrived at that the faint trace of hæmolysis observed in the control and most

of the other tubes was due to spontaneous lysis of the corpuscles due to ageing. The results are recorded in Table VIII —

TABLE VIII

*Results of tests in blood broth containing 2.5 per cent washed cells set in McIntosh and Fildes' jar incubated at 37°C*

Days at which readings were taken —	Greig positive organisms			Greig negative organisms
	1	2	3	9
Number of strains showing hæmolysis —				
Complete	8	35	36	*
Partial	26	1		2
Trace				1
Nil				23

\* There was a faint trace of hæmolysis in all the negatives as well as the saline control

The results of blood broth set under vaseline seal are given in Table VIII-a —

TABLE VIII-a

*Results of tests in blood broth containing 2.5 per cent washed cells set under vaseline seal and incubated at 37°C*

Days at which readings were taken —	Greig positive organisms				Greig negative organisms							
	1	2	3	4	1	2	3	4	5	6	7	8
Number of strains showing hæmolysis —												
Complete	1	27	33	36								2
Partial	35	9	3						2	3	5	7
Trace									1	3	1	1
Nil					26	26	26	26	23	20	20	16

N.B.—The control tube (uninoculated) began to show trace hæmolysis on the sixth day and partial hæmolysis on the eighth day but was found sterile on plating

The results are similar to those enumerated above. Blood-broth cultures of Greig-negative organisms are all of them negative up to the fifth day and thereafter there are some positives but the control also shows a certain amount of hæmolysis from the sixth day.

*C Effect of restriction of oxygen supply when the test is set*—Cultures were grown aerobically for 1, 2, 3, 5, 10 and 15 days and the test set under vaseline seal. The results are given in Table IX.

TABLE IX

*Results of tests in cultures in fluid media incubated at 37°C aerobically and test set at 37°C under vaseline seal*

Hours at which reading was taken —	24						48						72					
Age of cultures in days —	1	2	3	5	10	15	1	2	3	5	10	15	1	2	3	5	10	15
<i>Greig positive organisms</i>																		
Number of strains showing hæmolytic —																		
Complete	35	31	24	8	5	4	35	32	29	10	5	5	35	30	32	14	6	7
Partial		3	7	16	2	8	1	3	7	19	16	15	1	8	4	20	28	28
Trace	1	2	5	1	2	1		1		1	9	8		1		2	2	1
Nil				11	27	23				6	6	5						
<i>Greig negative organisms</i>																		
Complete						1						3			2			7
Partial						6			4	4	22	20			6	14	25	19
Trace			1			5			5	4	3	3			1	2	1	
Nil	26	26	25	26	26	14	26	26	19	18	1		26	26	17	10		



which exist, presumably comparable one with the other with any one concentration of blood cells. The numbers of cultures reacting in the series are, therefore, an indication of the relative sensitivity of the two methods. This matter will be referred to later.

In blood broth all Greig-positive organisms showed hæmolysis in 24 hours and all Greig negatives failed to show it. On further incubation six Greig-negative organisms showed lysis in 48 hours, 19 in 72 hours, 22 in four days and the whole 26 of the series in five days.

*Effect of setting the test at 12°C* —The results are given in Table XII —

TABLE XII

*Results of tests in cultures in fluid media grown at 37°C aerobically  
and test set at 12°C aerobically—blood concentration  
0.5 per cent*

Hours at which reading was taken —	Greig positive organisms								
	24			48			72		
Age of cultures in days —	1	2	3	1	2	3	1	2	3
Number of strains showing hæmolysis —									
Complete	14	27	16	14	32	17	15	32	18
Partial	7	8	4	16	4	3	17	4	2
Trace	15	1		6			4		1
Nil			16			16			15

All Greig-negative organisms failed to show lysis. The results with Greig-positive organisms show that as compared with 2.5 per cent blood cells the test records fewer positive reactions.

*Effect of the restriction of the oxygen tension* —Greig-positive organisms were tested by growing at 37°C under vaseline seal and setting test at 12°C aerobically. Greig-negative organisms were tested by growing at 37°C under vaseline seal and setting at 12°C under vaseline seal.

Greig negatives did not produce any lysis The results of Greig positives are given in Table XIII —

TABLE XIII

*Results of tests in cultures of Greig-positive organisms in fluid media grown at 37°C under vaseline seal and test set at 12°C aerobically—blood concentration 0.5 per cent*

Hours at which reading was taken —	24			48			72		
Age of cultures in days —	1	2	3	1	2	3	1	2	3
Number of strains showing hæmolytic —									
Complete	14	28	30	23	29	34	27	29	34
Partial	17	4	5	10	6	1	6	6	1
Trace	3	3		2	1		2	1	
Nil	2	1	1	1		1	1		1

In blood broth Greig-positive organisms showed complete hæmolytic after 24 hours' growth The results of Greig-negative organisms are shown in Table XIII-a —

TABLE XIII-a

*Results of tests in blood broth containing 0.5 per cent washed cells incubated at 37°C under vaseline seal—blood concentration 0.5 per cent*

Days at which reading was taken —	Greig negative organisms		
	1	2	3
Number of strains showing hæmolytic —			
Complete			7
Partial		7	7
Trace		2	8
Nil	26	17	4

Greig positive organisms showed complete hæmolytic after 24 hours' incubation

The restriction of oxygen supply by these methods will enormously improve the ease with which results can be read as there is no trouble from fading etc of the hæmoglobin

The results when the cultures were grown aerobically and the tests set under vaseline seal are given in Table XIV

TABLE XIV

*Results of tests in fluid media of cultures grown at 37°C aerobically and test set under vaselne seal at 37°C—blood concentration 0.5 per cent*

Hours at which reading was taken —	Greig-positive organisms									Greig negative organisms								
	24			48			72			24			48			72		
Age of cultures in days —	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Number of strains showing hemolysis —																		
Complete	7	36	5	25	36	19	28	36	24		1		4	3	15	17	14	23
Partial	25		13	11		8	8		8	2	1	12	12	14	9	4	10	3
Trace	4		4			3			2	8	2	8	5	3		1	2	
Nil			14			6			2	16	22	6	5	6	2	4		

The above results again emphasize the reduced sensitivity of 0.5 per cent cell concentration to the action of Greig-positive organisms in early readings but increased sensitivity in late readings to the action of Greig-negative organisms

*Effect of chemical reducing agents*—This was investigated in exactly the same way as with 2.5 per cent cells. The results were of the same order. All Greig-positive organisms showed a high degree of hæmolytic activity even in three-day cultures but the results with Greig-negative strains were similar to those obtained in the absence of reducing agents

TABLE XV

*Results of tests in fluid media of cultures grown at 37°C aerobically and chemical reducing agents added before test is set under vaselne seal at 37°C—blood concentration 0.5 per cent*

Hours at which reading was taken —	Greig positive organisms									Greig negative organisms								
	24			48			72			24			48			72		
Age of cultures in days —	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Number of strains showing hemolysis —																		
Complete	35	36	32	36	36	35	36	36	35		9	10	1	20	21	12	21	23
Partial	1		4			1			1	3	5	6	6	1	1	3	1	
Trace										2	4	6	7		1	3	1	
Nil										21	8	4	12	5	3	8	3	3

The decreased sensitivity of 0.5 per cent cells as compared to 2.5 per cent cells was confirmed by several titration experiments of which the following is an example

TABLE XVI

*Results of experiments done with 24-hour cultures of two Greig-positive organisms using washed cells in 2.5 per cent and 0.5 per cent concentration test set at 12°C*

Cultures		Culture dilution				
		1/2	1/5	1/10	1/20	1/40
Culture MW 136						
<i>Hæmolysis</i> —						
1 hour reading	Cells 2.5 per cent	C	C	C	C	C
	" 0.5 "		P	C	P	P
2 hour reading	" 2.5 "	C	C	C	C	C
	" 0.5 "		C	C	C	C
Overnight reading	" 2.5 "	C	C	C	C	C
	" 0.5 "	P	C	C	C	C
Culture El Tor 103 Celebes						
<i>Hæmolysis</i> —						
1 hour reading	Cells 2.5 per cent	C	C	C	C	C
	" 0.5 "					
2 hour reading	" 2.5 "	C	C	C	C	C
	" 0.5 "	tr	C	C	C	P
24 hour reading	" 2.5 "	C	C	C	C	C
	" 0.5 "	tr	C	C	C	P

C = Complete hæmolysis

P = Partial hæmolysis.

tr = Trace hæmolysis

= No hæmolysis

Culture MW 136 was one that retained its early hæmolytic activity unimpaired in 15 days' aerobic cultures

El Tor Celebes 103 was one that lost this activity after the third day of incubation

In the 0.5 per cent series very little fading of the lysed hæmoglobin occurred with the former strain, while marked or complete fading occurred from the first hour onwards with the second

The above hæmolytic strains which differ in character\* both show a decreased activity with respect to red cells in a concentration of 0.5 per cent. There is to some extent a zone phenomenon as it is most evident when undiluted cultures are used, i.e. when there is a final concentration of the culture of  $\frac{1}{2}$ .

*Action of antihæmolysin sera*—Potent antihæmolysin sera against two Greig-positive cultures were raised from rabbits by the method advised by Goyle (1938). These were employed to test their effect on the hæmolysis produced by Greig-positive and Greig-negative organisms. The titre of these sera against the homologous strain were 80 and 10. The organisms selected for injection into the rabbits were (1) an organism which in broth cultures was capable of maintaining its hæmolytic activity over 15 days or longer, (2) an organism which rapidly lost this power after the third day of growth. No qualitative differences in these sera were detected when tested against a number of Greig-positive strains. No preservative disinfectants were employed. The results recorded below are almost all of them with the more potent serum.

*Technique*—To nine drops of culture was added one drop of antihæmolysin serum (and in controls one drop of normal rabbit serum or one drop of saline). Tubes were incubated for one hour at 37°C for interaction if any to take place. Then ten drops of a 5 per cent goat's red cell suspension were added. Tubes were incubated at 37°C for three days. The final dilution of the serum was therefore 1 in 20. Tests were also put up with young cultures up to three days' growth but with a serum dilution of 1 in 4 (final concentration). Table XVII shows the results in the case of Greig-positive organisms.

While the normal rabbit serum was without effect on the results, no hæmolysis occurred in the presence of the immune serum with a concentration of  $\frac{1}{4}$ . When the serum content was reduced to 1/20, hæmolysis was able to occur in certain strains in the presence of immune serum, but when reduction of hæmolysin content was effected, as by using 4-, 6-, or 13-day cultures, the hæmolytic action was specifically inhibited. One strain which showed a very small hæmolytic titre was inhibited by both normal rabbit serum and immune rabbit serum.

The results with Greig-negative strains are given in Table XVIII.

In all tests except one there was no evidence of specific action of the antihæmolysin serum. Except in three-day readings of 15-day cultures hæmolysis was inhibited by both normal and immune rabbit sera, where hæmolysis took place in the presence of sera it took place equally in the presence of the normal and the immune serum.

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\* One strain was able to retain its hæmolytic power in air up to 15 days. The other lost it after three days' incubation.

TABLE XVII

*Results of tests of Greig-positive cultures in fluid media grown at 37°C aerobically and test set at 37°C aerobically in the presence of an antihemolysin serum*

Hours at which reading was taken —	Serum concentration 1/20																	
	24						18						72					
	1		4		0		13		1		4		10		1		4	
Age of culture in days:—	Sal		NRS		IRS		Sal		NRS		IRS		Sal		NRS		IRS	
	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS
Strain number	Sal		NRS		IRS		Sal		NRS		IRS		Sal		NRS		IRS	
	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS	Sal	NRS
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ = Complete or partial hemolysis

Sal = Saline control

IRS = Immune rabbit serum (antihemolysin).

— = Nil or trace hemolysis.

NRS = Normal rabbit serum control

TABLE XVII—concl'd

Hours at which reading was taken —	Serum concentration $\frac{1}{2}$											
	24						48					
	1			2			1			2		
Age of culture in days —	1			2			3			1		
	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS
Strain number	1			2			3			1		
	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS
1	+	+	—	+	+	—	+	+	—	+	+	—
2	+	+	—	+	+	—	+	+	—	+	+	—
3	+	+	—	+	+	—	+	+	—	+	+	—
4	+	+	—	+	+	—	+	+	—	+	+	—
5	+	+	—	+	+	—	+	+	—	+	+	—
6	+	+	—	+	+	—	+	+	—	+	+	—

+ = Complete or partial hemolysis  
 Sal = Saline control  
 IRS = Immune rabbit serum (antihemolysin)

— = Nil or trace hemolysis  
 NRS = Normal rabbit serum control

TABLE XVIII

*Results of tests of Gregy-negative cultures in fluid media grown at 37°C anaerobically and test set at 37°C. anaerobically in the presence of an antihemolysin serum*

Hours at which reading was taken —	72											
	18						0					
	13			15			1			2		
Strain number	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS	Sal	NRS	IRS
	+	+	+	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+

+ = Complete or partial hemolysis

Sal = Saline control

IRS = Immune rabbit serum (antihemolysin)

- = Nil or trace hemolysis

NRS = Normal rabbit serum control



*Action of cultures killed by formalin*—Douglas-broth cultures were treated with formalin in a concentration of 0.1 per cent and the tubes were incubated at 37°C for four hours. They were then tested for sterility. This exposure was found invariably to kill the cultures. A further sterility test was taken on completion of the test. The results are shown in Table XIX.—

TABLE XIX

*Results of tests of cultures in fluid media grown at 37°C aerobically and killed by 0.1 per cent formalin and test set at 37°C aerobically*

Hours at which reading was taken —	24						48						72					
Age of culture in days —	1	2	3	5	10	15	1	2	3	5	10	15	1	2	3	5	10	15
<i>Greig positive organisms</i>																		
Number of strains showing hæmolysis —																		
Complete	34	22	14	5	1		36	23	15	5	1		36	25	15	5	2	
Partial	2	2	1	1	2			3		1	2			1		5	6	1
Trace		2						3	5	12	9	3		10	19	25	22	9
Nil		10	21	30	33	36		7	16	18	24	33			2	1	6	26
<i>Greig negative organisms</i>																		
Complete																		
Partial									1				2	1	2	4	4	1
Trace	1			1		1	2	1	9	11	6	2	2	2	16	16	17	9
Nil	25	26	26	25	26	25	24	25	16	15	19	24	22	23	8	16	5	16

The presence of formalin has reduced the hæmolytic power of broth cultures, both of Greig-positive and Greig-negative organisms. Neglecting trace reactions a considerable number of partial reactions are shown in the 72-hour readings of Greig-negative organisms. Hæmolysis, therefore, by both Greig-positive and Greig-negative organisms can take place in the absence of viable forms of the organisms. A further attempt was made to try to eliminate any possible effects of the formalin and at the same time using non-viable cultures by freezing broth cultures. (It is a matter of experience that agar slopes of cholera vibrios, especially old agar slopes, are easily killed by cold.) Forty-eight hours' freezing

was, however, ineffective, while alternate freezing and thawing 11 times of a set of ten seven-day cultures, also proved ineffective. One culture was, however, rendered sterile. A further culture was, moreover, found sterile after eight days' growth by accident. Both these cultures (Greig-negative organisms) gave no reaction with 2.5 per cent red blood cells after 24 hours' incubation at 37°C but there was a trace reaction in 48 hours, and a partial reaction in 72 hours. The addition of 0.1 per cent formalin to one of the two cultures eliminated the partial reaction but did not prevent the trace reaction.

With L<sub>3</sub> candle filtrates of Greig-positive and Greig-negative organisms, it was found that, while the former retained their power of hæmolysing goat cells, the latter lost their activity on these cells. With either type of organism, care was taken to reject the first 20 c.c. to 25 c.c. of the filtrate before collecting the sample for test.

### DISCUSSION

A study of the above findings will leave no doubt that the distribution into two groups based upon the Greig test is supported in some degree by all the methods of test. It is certain that there is a difference of the conditions under which the hæmolysin is produced in the two groups. The two may be distinguished on a time factor in the production of hæmolysin, 'early hæmolysin' being defined as that which is produced within 24 hours' incubation from a culture of any age while 'late hæmolysin' is produced after 24 hours.

The characteristics of the early hæmolytic type are —

Hæmolysis is usually complete within 24 hours. In many cases it is so within a few minutes.

Reduction of the temperature at which the test is set to 12°C will diminish the production of hæmolysin but will not markedly affect it under the condition of test unless the culture is a very weak hæmolysin producer or has been reduced to a relatively inactive stage.

The production of the hæmolysin is very little affected by diminishing the oxygen supply, although the degree of growth in the culture is very slight as judged by opacity.

Most but not all of the cultures of three days' growth and upwards grown in air, partially or wholly, lose their power to cause lysis in 24 hours, but if the oxygen supply is restricted they maintain it up to 15 days or longer.

There is a high degree of consistency in the results of repeat examinations by the same methods.

Hæmolysis is specifically inhibited by an immune serum raised by injection of the hæmolysin into rabbits.

The hæmolysin is not removed by filtration through an L<sub>3</sub> candle.

The characteristics of the late hæmolytic type are —

Hæmolysis produced is usually partial and with adequate blood concentration never occurs in 24-hour readings

Reduction of temperature to 12°C at which the test is set completely abolishes the power of these strains to produce hæmolysis

The production of hæmolysis is with adequate blood concentration largely abolished by restricted oxygen supply represented by the use of vaseline seal and completely by strict anærobiosis as by growth in McIntosh and Fildes' jar

Cultures up to 15 days' growth are well able to produce their effect if incubated in the presence of red cells for two to three days such cultures, however, never produce rapid complete lysis Their hæmolytic power is not destroyed in the presence of oxygen

The degree of inconsistency between repeat tests is much higher than with the 'early hæmolytics'

The hæmolytic power is not specifically inhibited by an immune serum prepared by injecting the hæmolysin of an early hæmolytic type organism into rabbits.

The hæmolysin is removed by filtration through an L<sub>3</sub> candle

Whether therefore the actual mechanism of lysis is qualitatively the same in the two groups or not the points of difference are sufficiently great to establish beyond dispute the difference based on the Greig test

The question may arise as to what is the method of choice to demonstrate for routine work the difference between the two groups In the opinion of the authors the use of reduced temperature used in the Greig test is likely to bring certain strains that would otherwise be classed as early hæmolytic into the opposite group In the series chosen by the authors this has not occurred (one strain labelled El Tor of considerable age showed no relationship by any test with the 'early hæmolytic' type) but when dealing with old cultures or those with a low lysin titre the mistake might occur The distinction between the groups can be made by incubating at 37°C In addition by utilizing this temperature it is possible to make use of blood broth which is reliable and saves a great deal of trouble When it is intended to take readings after 24 hours' incubation the use of vaseline seal will abolish the interference from alterations in the lysed hæmoglobin The use of blood plates is reliable provided the appearances at the different stages are known

With regard to the nature of the active principle all that can be pointed out at the present stage is that very different conditions are necessary for the production and maintenance of the hæmolysin in the two different types of organism What these conditions are has already been enumerated It was thought for some time that the 'late' hæmolytic effects were likely to be associated with the power of the organisms to multiply actively The fact, however, that both formalin-killed

Greig-negative cultures and in two cases cultures killed by other means were still able to produce hæmolysis is against this fact. The presence of formalin is, however, associated with a partial diminution in the hæmolysing power of both types of culture which may be the result of interaction either with the hæmolysing agent or with the corpuscles, so that the effect of the growth of the culture in the production of late hæmolysis has not been eliminated. The action of the specific immune sera used is against the idea that the hæmolysing agents are the same, the proof is not complete. Very accurate titration experiments might possibly throw some light on this aspect of the problem.

It will be noted that late hæmolysis is also produced by early hæmolysin producers, for old cultures in which the capacity to produce early hæmolysis had disappeared, can still produce late hæmolysis, which fact is in itself against any unity hypothesis.

It seems likely that early and late hæmolysis are different from each other and are identical with the true hæmolytic and hæmodigestive ferments described by van Loghem (1913) in the case of solid media. The complete elimination of the cleared and green zone on blood agar plates incubated anærobically in the case of Greig-negative organisms and the presence of only a clearing without the greening in the case of Greig-positive organisms support this idea.

#### SUMMARY

1 The action on goat's red cells of *V. cholerae* and El Tor type vibrios grown in isotonic Douglas broth have been tested by various methods. The organisms were grown in this medium for periods from 1 to 15 days both in the presence of air and by restricting oxygen by means of vaseline seal and by the use of the anærobic jar. The growths were tested on goat's cells by incubating the tests at 37°C and 12°C in the presence of air, by restricting oxygen by means of vaseline seal and the anærobic jar, and after addition of chemical reducing agents. Both 2.5 and 0.5 per cent concentrations of the cells were employed. Results are recorded of growing the strains in blood broth of the same concentration and also on 10 per cent blood agar under aerobic and anærobic conditions.

2 The results show that the Greig test is supported in some degree by all the methods of test.

3 The strains can be divided into two groups: the 'early hæmolytic' and the 'late hæmolytic', corresponding to Greig-positive and Greig-negative organisms. In the former group, hæmolysis is usually complete within a few minutes to 24 hours, is not markedly affected by performing the test at 12°C or under reduced oxygen tension and production of the hæmolysin is not affected by exclusion of oxygen. In the latter group hæmolysis is usually partial, hardly occurs in 24 hours and is abolished when the test is performed at 12°C or under restricted oxygen supply.

4 Antihæmolytic sera prepared from Greig-positive organisms have a definite specific neutralizing effect on the hæmolysin of the early hæmolytic group. No similar effect has been demonstrated in the late hæmolytic group.

5 Formalin-killed cultures of both groups are still able to produce their effect on goat's red cells though this is markedly reduced.

6 The 'early' and 'late' hæmolysins are very likely identical with the hæmolysin and the hæmodigestive ferments respectively, described by van Loghem in respect of solid blood media.

#### ACKNOWLEDGMENTS

Our thanks are due to Dr C E de Moor, Macassar, for the supply of Celebes strains, to Major C L Pasricha, I.M.S., for some of the classical El Tor strains and to the Director, Central Research Institute, Kasauli, for the preparation of two antihæmolytic sera.

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## THE PREPARATION OF A VACCINE AGAINST BACILLARY DYSENTERY

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[Received for publication, December 31, 1941]

THE Annual Report on the health of the Army in India for the year 1939 gives the incidence of bacillary dysentery among British troops as 47.2 per mille and states that 'there is little tendency to reduction in either British or Indian figures'. A comparison is drawn with the enteric group of diseases whose incidence has been so greatly reduced in the last 20 years—this reduction being attributed to a considerable extent to preventive inoculation. In view of this position it has been considered desirable to re-investigate the possibilities of prophylactic inoculation with vaccines prepared from dysentery strains and the studies now reported have been made with the object of determining the antigenic and protective value of vaccines consisting of different fractions or suspensions detoxicated by various methods.

As over 75 per cent of infections in India and elsewhere are of the Flexner type and the problem at issue is more simple than in Shiga infections it is proposed to consider first the preparation of Flexner vaccine.

### FLEXNER STRAIN VACCINES GENERAL CHARACTERISTICS OF GROUP

For the purpose of establishing general principles applicable to the preparation of a vaccine, a series of six strains of the Flexner V antigenic type, recently isolated from dysentery cases, were obtained from the Enteric Laboratory, Kasauli. The studies made on these strains were for the purpose of determining —

- (a) The regularity of the characters of the strains of similar antigenic type
- (b) Suitable methods for their maintenance
- (c) Resistance to heat and chemical disinfectants

Six Flexner V strains were compared *inter se*. In morphology, staining and cultural characters all strains were true to textbook description. Viability did not exceed 2 months on Douglas agar at room temperature and horse-blood agar is recommended for their maintenance with monthly sub-culture. An apparent dissociation occurred in two of the strains but this was limited only to a difference in size of colony and was found to be exaggerated by the use of egg-cleared Douglas agar. When clearing was carried out without egg these appearances no longer occurred. The small and large colonies were serologically identical.

#### FERMENTATION AND BIOCHEMICAL REACTIONS

The usual carbohydrates were employed and reactions observed over a period of 7 days —

Glucose	Lactose	Mannite	Maltose	Peptone water (Indol)	Milk
Acid	0	Acid	$\frac{\text{Acid}}{0}$	$\frac{+}{0}$	No change

Many strains recently isolated were examined to determine if a parallel existed between biochemical and serological affinities, with negative result. It was revealed, however, that the most active sub-group was the so-called Y strain and this usually fermented maltose and produced indol although a few Y strains were found which were not active in these respects.

#### RESISTANCE TO HEAT AND CHEMICAL DISINFECTANTS

Two of six strains examined showed a surprising degree of resistance to heat, surviving 60°C for 1 hour, while the remaining four survived 60°C for half an hour as shown in a subsequent protocol and confirmed by Major R. N. Phease, R.A.M.C., at the Enteric Laboratory (see *Appendix I*).

This finding is at variance with textbook statements probably based on the study of strains frequently sub-cultured. Roelcke (1940) reports similar heat-resistant characters in the Sonne group.

To chemical disinfectants all Flexner V—Z and Flexner Boyd strains are susceptible in low concentrations of disinfectants used: phenol 0.5 per cent, merthiolate 1 in 10,000, alcohol 75 per cent, formalin 0.4 per cent, 10 per cent bleaching powder (25 per cent free Cl<sub>2</sub>) all sterilized opacities up to No. 7 Brown's tube on 24 hours' contact.

*Exotoxin production* — Strains of V, W, Z and 170 were grown for varying periods up to 7 days in broth and their filtrates injected intraperitoneally in quantities of 5 c.c. to rabbits, without evidence of exotoxin content.

#### DETERMINATION OF RELATIVE TOXICITY OF STRAINS

Eighteen-hour agar cultures suspended in 0.5 per cent carbol-saline, opacity No. 3 Brown's tube, prepared in parallel with known strains of

*Bact dysenteriae* (Shiga) and *Bact typhosum* (enteric Laboratory V<sub>1</sub> I strain) were injected intraperitoneally to 20-gramme mice with the results given in Table I —

TABLE I

Strain	Dose in milligrams					
	0 04	0 08	0 12	0 16	0 2	0 32
<i>Bact dysenteriae</i> (Flexner) V <sub>1</sub>	S	S	S	D36	S	D24
" " V <sub>2</sub>	S	S	S	S	S	D29
" " V <sub>3</sub>	S	S	S	S	D25	D12
" " V <sub>4</sub>	S	S	S	D36	D46	D24
" " V <sub>5</sub>	S	S	S	D40	S	D36
" " V <sub>6</sub>	S	S	S	S	S	D18
<i>Bact typhosum</i>	S	D25	S	D23	D32	
<i>Bact dysenteriae</i> (Shiga)	S	D107	D105	D91	D68	
<i>Bact Sonne</i>	S	S	S	S	S	
<i>Bact Schmitz</i>	S	S	S	S	D50	
170 (Boyd)	S	S	S	S	S	
88	S	S	S	S	D36	

S = Survived 48 hours

D = Death in hours

The V strains *inter se* showed minor degrees of difference of toxicity, the average lethal dose (a l d) required to kill in 48 hours varying from 0 16 mg to 0 32 mg. Batches of 5 and 9 mice were also employed and yielded similar results an a l d of 0 16 mg to 0 32 mg being the dose required to kill the majority of the animals in 48 hours as shown in Table II. Comparative figures for *Bact shiga* and *Bact typhosum* were 0 04 mg and 0 08 mg although it should be observed that death in Shiga-inoculated mice is much delayed often up to 3 or even sometimes 7 days



A repeat test on larger number of mice gave confirmatory results with the V strain as shown in Table II —

TABLE II

Strain	Number of mice	Dose, mg	Death	Survival
V <sub>4</sub>	5	0.12	0	5
	5	0.16	0	5
	5	0.2	1	4
	5	0.22	0	5
	5	0.24	3	2
V <sub>1</sub>	9	0.24	5	4
	9	0.28	5	4
	7	0.32	5	2
V <sub>6</sub>	9	0.24	1	8
	9	0.28	2	7
	7	0.32	4	3

## DETOXICATION OF FLEXNER STRAINS

The effect of treatment with formalin, merthiolate, alcohol and calcium hypochlorite in various strengths on the toxicity of suspensions of the V<sub>4</sub> Flexner strain was tested with the result shown in Table III —

TABLE III

Strain V <sub>4</sub>	Number of mice	Dose, mg	DEATH	SURVIVAL
			48 hours	
(a) Formalized 0.4 per cent suspension	9	2.5	0	9
(b) Merthiolate (1:10,000) suspension	9	1.25	3	6
(c) Phenol 0.5 per cent, 1 month contact (control)	9	0.25	4	5
(d) Phenol 0.5 per cent, 24 hours' contact (control)	9	0.25	3	6

(1) Further experiment showed the optimum concentration of formalin to be 2 per cent with a period of contact at 37°C for 3 weeks with subsequent suspension of centrifugate in 0.5 per cent phenol. Table III shows a degree of detoxication 10× which was not always obtained with all strains used, but a range of detoxication of from 4× to 10× was established for formalin.

(2) Merthiolate also detoxicated suspensions but never to the same degree as formalin.

(3) Bleaching powder in certain strengths having 25 per cent free chlorine also detoxicated suspensions but as the resulting product suspended in 0.5 per cent phenol was found to have lost much of its antigenic property there seemed to be no need for further investigation.

(4) Alcohol 75 per cent was also tried and produced a measure of detoxication but, like merthiolate, not to such a degree as formalin, and its antigenic property was not so definite, although better than with bleaching powder.

#### VIRULENCE OF LIVING CULTURES OF FLEXNER GROUP

Six V, one W and one Boyd 170 strains were examined and compared with *Bact. dysenteriae* (Shiga) and *Bact. typhosum* of known virulence. Felsen (1938) records 3½ millions as the minimum lethal dose (m.l.d.) for 15-gramme mice. French workers (Thibault and Rist, 1940) regard this American strain as exceptionally virulent and such levels were not obtained in our investigation.

Using over one hundred mice, the virulence dose (i.e. the a.l.d. killing 50 per cent of 20-gramme mice in 7 days by intraperitoneal inoculation) was found to be from  $250 \times 10^6$  to  $360 \times 10^6$  organisms. Comparative figures for *B. typhosum* and *B. dysenteriae* (Shiga) were  $215 \times 10^6$  and  $12 \times 10^6$  respectively.

It will be seen that this dose of living Flexner bacilli approaches or even on occasion exceeds the lethal dose of dead organisms, a finding not unexpected with organisms of relatively low virulence. Table IV exemplifies one of a number of virulence tests —

TABLE IV

Strain	Number of mice	Dose, c.c. (No. 6 opacity)	Viable counts	MORTALITY		Survival to 28 days
				48 hours	28 days	
V <sub>4</sub>	9	0.3* ( $810 \times 10^6$ )	$270 \times 10^6$	1	1	8
	9	0.4 ( $1,080 \times 10^6$ )	$360 \times 10^6$	5	5	4
	9	0.5 ( $1,300 \times 10^6$ )	$450 \times 10^6$	6	8	1
	8	1 ( $2,696 \times 10^6$ )	$910 \times 10^6$	8	8	0

\* Saline suspension of 18 hour growth—peritoneal inoculation.

In view of the low virulence level, Rake's (1935) mucin method was used as in *meningococcal* investigations. In this method the infecting dose in mucin is injected intraperitoneally and its use avoids the risk of the lethal action being due to the toxic effect of the inoculum rather than to multiplication and invasion by the organisms. Early results were inconsistent and unsatisfactory, but when the viscosity of the mucin was increased to 8 per cent and the pH to 7.3, the method proved most useful and accurate when the one strain of Flexner was used throughout.

The aid by Rake's method of the particular V<sub>4</sub> Flexner strain used was one million organisms checked by viable count, and in subsequent protection experiments, immunized animals resisted 50 a.i.d. and 100 a.i.d. ( $50 \times 10^6$  to  $100 \times 10^6$ ) while controls succumbed to the  $1 \times 10^6$  dose in mucin.

#### THE PREPARATION OF A SUITABLE ANTIGEN

Having ascertained the levels of toxicity and virulence of the Flexner group work was directed towards the selection of an antigen which would immunize and protect animals. The following were examined in turn—

(1) *Suspension of whole cells*—Eighteen-hour agar-grown bacilli were suspended in 2 per cent formal-saline and kept at 37°C for 3 weeks and thereafter the centrifugate was suspended in 0.5 per cent phenol. Agglutinin titres in rabbits after injection of three doses totalling 1 mg. reached 1 in 20,000 to 1 in 40,000 in 3 weeks without loss of weight, in a large number of rabbits. Batches of mice, to be described later, were immunized with this preparation with satisfactory results. Intradermal inoculation of guinea-pig and monkey showed mild inflammatory skin reaction without necrosis. Monkeys were injected with 0.5 mg. to 1 mg. without rise of temperature or loss of appetite and agglutinin titres of 1 in 125 to 1 in 500 thereafter obtained from them.

(2) *Lipoid antigen*—This is an acetone extract, acetone being subsequently distilled off at 40°C and remaining fraction Seitz filtered. It contained 20 mg. of solids per c.c. and was non-toxic to mice in doses of 1 c.c., rabbit agglutinin titres of 1 in 20,000 were easily obtained, active and passive tests in mice against 50 a.i.d. (mucin technique) were satisfactory.

Further treatment of the lipid by ether extraction revealed that the fatty acid fraction was inert as an antigen while the remaining fraction which was merely protein autolysate gave the following chemical tests: Xanthoproteic positive, biuret positive, Molisch a faint positive, while Millon's test and Benedict test and the test for sulphur, were negative.

(3) '*Acetone cells*'—This is the fraction remaining after treatment of the culture with acetone and the method followed was described by Felton and Wakeman (1937). The residue was dried, weighed and suspended in 0.5 per cent phenol. It showed a toxicity similar to whole cells, weight for weight, but was not detoxicated with formalin to the same degree as original cells—0/5 mice survived 1.25 mg. It produced agglutinin titres in rabbits to 1 in 40,000, and in an active protection test mice survived 50 m.i.d. of living culture in mucin.

- (4) Bleach treated cells
- (5) Supernatant washings [ectoantigen of Ferry and Fisher (1924)]
- (6) Formalized autolysates were also examined but each fell short in some essential of a satisfactory antigen standard

Further chemical extraction of active antigenic principles was not considered a practical proposition for the preparation of vaccines on a large scale

#### ANIMAL IMMUNIZATION

The antigen used for the following protection tests was formalized whole cells prepared as described —

(A) *Active immunity*—Thirty mice immunized by subcutaneous inoculation of doses of 0.025 mg and 0.05 mg with 4-day interval. Resistance was tested 10 days later against 100 m.l.d. using mucin technique

30 mice immunized	Test dose Flexner $V_4$ $80 \times 10^4$ in 0.1 c.c. saline added to 0.5 c.c. 8 per cent mucin intraperitoneally	Mortality 28 days 0/30
30 mice controls	$80 \times 10^4$ in 0.1 c.c. saline added to 0.5 c.c. 8 per cent mucin intraperitoneally	16/30*

\* Organisms isolated from heart blood

Several tests similar to above were carried out with similar results

The following table shows non-specific immunity in relation to the general problem —

Number of mice	Antigen used to immunize	Dose living culture Flexner $V_4$	MORTALITY		
			24 hours	48 hours	TOTAL
30	0.025 mg * Flexner $V_4$ 0.05 4-day interval	$12 \times 10^4$	1		1/29
30	0.008 mg * <i>B. typhosum</i> 0.004 4-day interval	$12 \times 10^4$	2	6	9/29
10	Control (no immunization)	$12 \times 10^4$	1	8	9/10
10	Control	$12 \times 10^4$	2	3	5/10

(a) 1 m.l.d. determined previous day =  $12 \times 10^4$  Flexner  $V_4$  in 8 per cent mucin

(b) \* being 1/10th a l.d.

(B) *Passive immunity*—Rabbits' immune serum (0.5 c.c.) inoculated intraperitoneally to 20-gramme mice 1 hour before the toxigenic dose protected

against 100 m l d (mucin technique) and against 5 m l d in straight inoculation procedure

Monkeys immunized by 0.5 mg and 1.0 mg showed an average agglutinin titre of 1 in 250 and the following is an account of this immunological experiment —

#### PROTECTION EXPERIMENTS IN THE MONKEY

Much experimental work has been done on immunization against dysentery infections. Most of the records have been based on the results obtained in the usual small laboratory animals in which the production of agglutinins and anti bodies demonstrated in different ways has been observed. In these, protection either active or passive, has been obtained against a parenteral infection of the living homologous strain.

While such work can give indications of the lines which may be suitable for the production of a vaccine, the evidence obtainable is not directly applicable to assessing the value of a vaccine against the usual method of oral infection which occurs in dysentery.

Our earlier work was carried out along such orthodox lines making use of mice and rabbits in assessing the value of various proposed antigens. It has been shown that a formalized suspension having a comparatively low toxicity will produce a high level of agglutinins in animals and man and protection against intraperitoneal infection in mice.

The sera of immunized animals had also a high protective value.

In the course of the investigation, tests were undertaken in monkeys to assess the probable toxicity and the reactions that are obtained by different doses as a guide to the selection of a dose for man.

A spontaneous Flexner infection occurred in one of the monkeys and it appeared that these animals would be suitable for immunological study.

Monkeys (*Macacus rhesus*) of 3 kilo to 4 kilo body-weight, in three successive batches of eight and one of sixteen, making a total of forty animals, were used. Of these 50 per cent were controls and the experiments extended over a period of 4 months.

The infecting organism, closely related to the human Flexner strain (see *Appendix II*), was obtained from the above spontaneous infection in which the animal died after an illness of 3 days. The post-mortem examination revealed a picture similar in every way to that seen in man. The large intestine bore the brunt of the attack and showed from anus to two inches beyond the ileocaecal valve an acute hæmorrhagic enteritis, the normal bowel flora being replaced by almost pure culture of *B. dysenteriae* (Flexner). Such appearances were very similar in all three post mortems made. Blood cultures were invariably sterile.

A careful search of stools of 12 monkeys just received from Delhi, plated on MacConkey's medium, resulted in the isolation of the pathogenic strain on two

occasions only, but this was sufficient to indicate its presence in these animals under natural conditions. By contrast, during the feeding experiments there was no difficulty in recovering the organism from protected and unprotected animals alike. The sera of all the animals were examined before inoculation for natural agglutinin level. This never exceeded 1 in 25, which was unexpectedly low as these monkeys were not from the virgin jungle but from the suburbs of the Punjab villages and lived in close association with man.

Each batch of monkeys was immunized by inoculation of 0.5 mg followed in 4 days by 1 mg of detoxicated Flexner V vaccine with which the work in the preparation of a vaccine had been done. In terms of Brown's tubes, each c.c. contained 1 mg giving a No. 7 opacity representing 3,148 millions per c.c. This immunizing dose was given to all subsequent batches. The animals showed very little discomfort, never missed a meal and had no significant rise of temperature. Ten days after the second dose the inoculated and non-inoculated animals were fed by giving each a small piece of banana impregnated with dysenteric mucus from the monkey which died, re-inoculated with a 4-mm platinum loopful of first sub-culture of the monkey strain.

Such feeding was given on an empty stomach each morning for 7 days. Other observers, e.g. Dack and Petran (1934), Preston and Clark (1938), have used large amounts of material (e.g. whole growth from a Petri dish) to infect monkeys, a procedure which pre-supposes difficulty and can have only an experimental outlook.

Our efforts were designed to imitate infection under natural conditions and only a small dose of infecting material was given. By the third day of feeding, in each batch infection had occurred among the controls and in the whole series two of the six dysenteric animals died after an illness of 3 to 4 days, the homologous organism being recovered from the dysenteric stools.

Batch	Number immunized.	Case of dysentery	Number of controls	Case of dysentery
1	4	0	4	1
2	4	0	4	1
3	4	0	4	1
4	8	0	8	3*

\* 2 deaths

No immunized animal developed dysentery. The sera of these showed agglutinin titres of 1 in 125 to 1 in 500 and 0.5 c.c. protected 15-gramme mice (10/10) against 4 a.l.d., i.e. against 1 c.c. containing  $3,146 \times 10^6$  living Flexner V<sub>4</sub> strain organisms, while a control batch (4/5) succumbed to 0.25 c.c. in 48 hours. Protective serum as in all passive immunity tests was introduced intraperitoneally 1 hour before the bacterial inoculum.

Although the number of animals employed would appear small the results are statistically significant

#### THE PREPARATION OF FLEXNER VACCINE

The vaccine suggested for use against Flexner infections is a 2 per cent formalin detoxicated trivalent antigen consisting of —

Flexner V	0.5 mg	} or $\frac{1 \text{ mg per c c}}{3,146 \times 10^6 \text{ per c c}}$	No 7 opacity Brown's tube
W	0.25 mg		
Z	0.25 mg		

This is made from very recently isolated strains (3rd or 4th sub-culture from patient) grown for 18 hours on Douglas agar in 20-ounce McCartney bottles, washed off with 2 per cent corrected formalin and placed in the incubator at 37°C for 3 weeks. Each strain is then centrifuged and the centrifugate is suspended in 0.5 per cent carbol-saline to No 7 opacity, thereafter they are mixed in requisite proportions and should pass sterility, toxicity, and agglutinogenic tests

1 In toxicity tests 0.75 mg fails to kill any of five 20-gramme mice employed in batch tests

2 As an agglutinin in rabbits 1 mg produces a titre of 1 in 20,000 in 10 to 21 days without material loss of weight to the animal

The vaccine in the doses indicated was tried in 20 human volunteers and did not produce any severe local or general reaction

#### THE PREPARATION OF A SHIGA VACCINE

Prigge (1941) states that effective protection against *B. dysenteriae* (Shiga), in his view, can only be obtained by injection of a preparation containing exotoxin and endotoxin. He inoculated 44 persons, 32 of whom showed significant anti-toxin averaging 1 International unit per c.c. 4 weeks after the last of three injections of one preparation. Other immunologists maintain that anti-bacterial immunity should be the chief aim.

In deference to the latter view the work done on Shiga followed very closely the technique of the Flexner investigation and may be summarized under the same headings

Five recently isolated strains were examined

**Toxicity**—Tests using 18-hour agar culture suspended in 0.5 per cent carbol-saline killed the majority of 20-gramme mice in 7 days with doses ranging from 0.04 mg to 0.08 mg, as compared with 0.16 mg to 0.32 mg for the Flexner strains

**Virulence**—The a.l.d. of live 18-hour culture in broth was 0.1 c.c., the majority of mice being killed in 7 days. The equivalent viable counts were from 10 to  $40 \times 10^6$  organisms per a.l.d. as compared with  $350 \times 10^6$  of Flexner strains

*Detoxication*—The choice lay between formalin 2 per cent and alcohol 75 per cent after trial of other possible detoxifying agents. In tests the majority of mice survived a 0.6 mg dose of formalin-detoxicated Shiga antigen and only 0.3 mg of alcohol-detoxicated substance. The reduction of toxicity with formalin was approximately ten times. The alcohol vaccine, however, produced a higher agglutinin titre in rabbits by intravenous injection and the serum of these animals protected mice better than formalin-antigen produced serum. A further experiment was done by inoculating *subcutaneously* 18 rabbits of 2 kilo body-weight with 0.43 mg of freshly prepared alcohol-detoxicated vaccine and 15/18 died within 7 days. In our tests it was found that the rabbit was particularly sensitive to the toxin of *B. dysenteriae* (Shiga). It was also found that alcohol-detoxicated vaccine preserved with phenol loses in potency after a period of 3 months.

Titres of 1 in 2,500 to 1 in 5,000 were obtained in rabbits by the intravenous route (total antigen given amounting to 1.5 mg), without loss of weight, enterotropic or neurotropic disturbance. The dose for the monkey 0.5 mg subcutaneously followed in 7 days by 1 mg seemed satisfactory, and was not too toxic and produced agglutinin titres of 1 in 50 to 1 in 500. Two monkeys having 1 in 500 titres examined after a period of 14 weeks showed titres of 1 in 250.

Intradermal injection in guinea-pigs resulted in a minimal degree of inflammation without necrosis.

Batches of mice were immunized subcutaneously by 1/10th (0.03 mg) of an ald of alcohol antigen given in two doses and 10 days thereafter their resistance was tested and compared with mice similarly inoculated at the same time with equivalent doses of other antigens as in the following protocol—

Number of mice.	Antigen used to immunize	Toxicogenic dose	MORTALITY		
			48 hours	7 days	TOTAL
18	Formalized whole cells	0.2 c.c. 18 hour broth culture— 0.5 c.c. mucin	6/18	6	12/18
18	Acetone cells	Ditto	14/18	2	16/18
18	Alcohol antigen	Ditto	6/18	0	6/18
9	Control (no immunization)	0.1 c.c. 18 hour broth culture— 0.5 c.c. mucin	9/9		9/9
9	Control „	0.01 c.c. 18 hour broth culture— 0.5 c.c. mucin	2/9	5	7/9



In this, and other tests using fewer mice, the alcohol-detoxicated vaccine would appear to be the best protective antigen

A Shiga vaccine, alcohol-detoxicated, preserved in 0.5 per cent phenol, is suggested for prophylaxis in man in doses of 0.43 mg and 0.86 mg or 0.5 cc and 1 cc of No. 6 opacity Brown's tube (2,777 millions per cc)

As opportunity offers, both vaccines (Flexner and Shiga) are being tried to determine local and general reaction and where possible the assessment of protective antibody

#### SUMMARY

It is considered that principles have been arrived at which will indicate suitable methods of detoxication of Flexner and Shiga vaccines without reducing antigenic value. Levels of dosage are indicated and a field trial of vaccine prepared on these lines is recommended.

Definite protective value is considered to have been shown in monkeys against Flexner infections by the natural oral route.

## APPENDIX I

It was established that many of the Flexner strains manifest an unusual degree of resistance to heat and Major R N Phease, R A M C, at the Enteric Laboratory, Kasauli, confirmed these results by the following method —

The strains tested were V<sub>2</sub> V<sub>3</sub> V<sub>4</sub> V<sub>5</sub> and V<sub>6</sub>, all of which had been isolated within the past 3 months

- Selected smooth colonies were inoculated on to agar slopes, incubated for 18 hours at 37°C and suspended in 5 c c sterile physiological saline

The suspensions were heated in a water-bath, the temperature of which was maintained at 60°C for 60 minutes The temperature was recorded (1) at the surface, (2) at the bottom and (3) in a separate control tube containing 5 c c saline

0.1 c c was sub-cultured into broth at 15-minute intervals up to 45 minutes

0.5 c c was sub-cultured into broth at the end of the period (60 minutes)

- Sub-cultures were made from the broth tubes after incubation for 24, 48, 72 and 96 hours

The results obtained were as follows —

Strain —	V <sub>2</sub>				V <sub>3</sub>				V <sub>4</sub>				V <sub>5</sub>				V <sub>6</sub>			
	15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60	15	30	45	60
24	+	-	-	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-	-	-
48	+	-	-	-	+	+	-	+	/	+	+	-	+	+	-	-	-	-	-	-
72	/	-	-	-	/	/	/	/	/	/	+	-	/	/	-	-	-	-	-	-
96	/	-	-	-	/	/	/	/	/	/	/	-	/	/	-	-	-	-	-	-

+ = Growth

- = Sterile

/ = Discontinued

## APPENDIX II

The characters of the monkey strain were in brief Morphologically indistinguishable from human Flexner strains, biochemically similar, fermenting mannite but not maltose and failing to produce indol in 7 days and serologically possessed of much group antigen of Flexner type

The following is an account of its antigenic relationship to the members of the Flexner-Boyd group, by Major Phaease —

' The apparent close relationship of the causal organism of natural dysentery in monkeys to members of the Flexner group, and in particular to the V strain, raises the question whether it is antigenically identical with any one of this group or whether it is a distinct type, sharing with the Flexner group a common group antigen

To determine this, cross agglutination and cross absorption tests were carried out, the results of which are outlined below —

For the purpose of the tests, all antisera used were standardized to a titre of 1 in 350.

For the absorption tests, 1 c c of serum was absorbed with 500,000 million organisms, the whole being made up to 20 c c with normal saline

Dreyer's technique was used throughout, primary readings being taken after 4½ hours in the water-bath at 50°C and final readings after 24 hours' incubation at 37°C.

TABLE I

[illegible]

TABLE II

[illegible]

The monkey Flexner bacillus is agglutinated to titre by the sera of strain V 88-Manchester-Newcastle and by the group strain Y. Sera of the other member possessing the group antigen agglutinate the monkey Flexner strain to 25 to 50 per cent of the titre.

On the other hand the monkey Flexner serum agglutinates to titre only the group strain Y. Strain V is agglutinated to 50 per cent of the titre, while no agglutination is obtained with strain 88.

*Cross absorption tests —*

1	Flexner V minus monkey Flexner	versus	monkey Flexner	<i>Nil</i>
	Flexner V	,	Flexner V	125
2	Strain 88	,	monkey Flexner	<i>Nil</i>
	Strain 88	,	strain 88	125
3	Monkey Flexner minus Flexner V	,	Flexner V	<i>Nil</i>
	Monkey Flexner	,	monkey Flexner	125
4	Monkey Flexner	strain 88	strain 88	<i>Nil</i>
	Monkey Flexner	,	monkey Flexner	250

CONCLUSION

The antigenic structure of monkey Flexner consists of a group element similar to that possessed by members of the Flexner-Boyd group, and a specific element peculiar to the strain.

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## A NOTE ON THE PRODUCTION OF *CLOSTRIDIUM* *PERFRINGENS* (TYPE A) TOXIN IN A MODIFIED LIVER AND VEAL DIGEST MEDIUM\*

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[Received for publication, December 26, 1941]

SOME of the media employed for the production of *Clostridium perfringens* (type A) toxin require the addition of a certain commercial peptone which is fairly expensive and sometimes difficult to obtain. For the study of 'botulinus' toxin Weinberg and Goy (1924) employed beef and liver digest medium in which no peptone was required to be added. Recently, Ipsen and Davoli (1939) used a similar medium for the production of *Clostridium perfringens* (type A) toxin. An attempt was, therefore, made to improve the technique of producing gas gangrene toxin in a modified liver and veal digest medium in which beef used by the above authors was replaced by young veal, a pepsin of higher strength and different concentration was used for digestion, glucose and some dried de-fatted meat was added to the basal broth and pH was adjusted to 7.8. The medium finally chosen, has given fairly consistent and reproducible results in the preliminary experimental production of *Clostridium perfringens* (type A) toxin of high potency, and the cost of the preparation is very much less than that of glucose-peptone-veal or beef-infusion broth‡. The results obtained in the above experimental work are reported in this paper.

### EXPERIMENTAL

**STRAIN** *Clostridium perfringens* (type A) of human origin, No. SR12 reported by Stewart (1940) to yield a potent toxin, was used throughout the experiment.

\* In conformity with the recommendations of a meeting held at the Biochemical Laboratory, Cambridge, on 5th April, 1941, as to the nomenclature which should be adopted for the toxins of *C. welchii*, type A, the toxin designated antigen I in this paper should be called *alpha toxin* and that designated antigen II should be called *theta haemolysin*. (See *Nature*, Volume 149, No. 3767, dated 10th January, 1942).—S C Seal

† The work was carried out in the National Institute of Health, Washington, D C (U S A), under the auspices of a Rockefeller Foundation Fellowship.

‡ Since this paper was written 60 litres of the medium have yielded a highly potent dry toxin.

MEDIUM The following medium was employed —

Fat-free veal	1 870 grammes
Beef liver (fresh)	450
Water	9 000 c c
HCl (concentrated)	75 „
Pepsin (1 10,000) (Difco)	0 25 gramme

To the above designated quantities of liver and veal, minced and mixed thoroughly with water, the required amounts of pepsin and concentrated HCl were added, and the preparation was heated in a water-bath at 48°C for 18 hours for digestion. During this time it was stirred at intervals. At the end of the period the temperature was brought to 85°C to stop digestion and the material was filtered, first through cheese-cloth and then through soft filter-paper. The pH was then adjusted to 7.8 using phenol-red as indicator and the broth was heated to 100°C for 10 minutes to induce precipitation. Finally, the material was passed through semi-hard filter-paper, distributed into flasks and sterilized at 110°C for 25 to 30 minutes. This broth served as the base for liver and veal digest medium.

For experimental purposes 25 grammes (10 per cent) of dried meat which had been extracted with water for the preparation of beef-infusion broth were measured into a 300-c c Erlenmeyer flask and covered with 250 c c of the digest broth. The mixture was heated in steam for about 10 minutes to balance the reaction which was then re-adjusted to pH 7.8, it was then sterilized at 110°C for 25 minutes. The reaction should actually be adjusted at a little higher level, pH 8.1 to 8.2, before autoclaving, as it had always been found that after the addition of meat and sterilization there was a drop in pH of about 0.2 to 0.4.

The flasks were inoculated with rapidly-growing five- to six-hour old culture of strain SR12, which had been passaged several times in whole-meat medium before the last culture, and they were incubated at 37°C for various experimental periods. At the end of these periods the pH was determined and the toxins were recovered by centrifuging the cultures and filtering them through Berkefeld N candles. Each sample was then tested for mouse m l d, hæmolytin titre (m h d) against sheep's red blood corpuscles, and opacity titre (m o d) (Nagler, 1939) against inactivated normal human serum. Borate-buffered-saline solution with gelatin was used as the diluting agent for toxin in these tests.

A preliminary study was made of several factors which might influence the nature of the toxin produced in liver and veal digest medium as base. The main findings were as follows —

1 *Influence of different glucose concentration on toxin products (Table I)* — Of the three different concentrations of glucose tried, namely 0.1, 0.25 and 0.5 per cent, the best result was obtained with 0.25 per cent glucose in the presence of 10 per cent dried meat. This medium in which the final pH was 6.2 yielded the most potent toxin showing a mouse m l d of 0.006 c c and an L + dose between 0.12 c c and 0.15 c c of the liquid toxin.

2 *Relative effect of dried meat and  $\text{CaCO}_3$  as buffer in the medium (Table I)* — This was studied in the presence of the three different concentrations of glucose mentioned above. The dried meat was added in 10 per cent and calcium carbonate in 2 per cent amounts. The latter was found optimum for pH 7.8 of the medium. The media containing  $\text{CaCO}_3$  were shaken as frequently as possible during the incubation period. The result showed that the buffer action of  $\text{CaCO}_3$  was as good as the dried meat but the yield of the toxin was about 10 times weaker with the former than with the latter.

3 *Effect of sodium thioglycollate in the medium (Table I)* — The substance was used in 0.05 per cent concentration in the presence of the three different amounts of glucose already mentioned, with or without meat. It showed a deleterious rather than a stimulating effect on the production of toxin.

4 *Comparative effect of dried and fresh meat with or without sodium chloride (Table I)* — The dried meat was added in 10 per cent amounts but the fresh meat, being heavier for the same volume, had to be added in double the quantities by weight (20 per cent). The media containing dried meat showed better results than those containing fresh meat but the effect of the salt was not clear. In some later experiments (*vide* Table V) it was noted that the addition of salt to the medium tended to improve the quality of toxin, particularly in regard to its hæmolysin content.

5 *Comparative efficiency of peptone-infusion broth and liver and veal digest broth in the presence of dried and fresh meat, with or without NaCl (Table II)* — The results showed that when the fresh meat was replaced by dried meat in glucose-salt-peptone-beef infusion the yield of toxin was better than that obtained in the same medium with fresh meat. Under the same conditions, however, liver and veal digest broth produced still somewhat better toxin than the infusion broth.

6 *Effect of varying the incubation period on the toxin production in liver and veal digest broth with and without dried meat and glucose (Tables III and IV)* — The readings were taken up to seven days beginning from 6 hours. The pH of the media containing glucose but no meat fell rapidly and reached the level of 4.8 in 12 hours, whereas, with meat added, the pH remained practically steady after it had reached the level near 6.0 in 16 hours rising slowly after 36 hours to 6.5 on the sixth day. In media containing meat but no glucose the fall of pH was still less marked. The peak of the toxin production (166 m.l.d. per c.c.) in media containing glucose was reached between the 16th and the 20th hour of incubation after which it began to fall at a slow rate up to 36 hours and then rapidly, more than 90 per cent of the toxin being destroyed by the fifth day. In media without glucose the hæmolysin titre was highest at 9 hours, while the peak of toxin production (100 m.l.d. per c.c.) was reached in 24 hours. After this period the potency fell practically at the same rate as in the other case. Thus, as observed in case of other media, glucose seemed to have a supplementary effect on the toxin production in the media under investigation.

7 *Comparative effect of dried meat, ether- and alcohol-extracted dried meat and dried bacto-beef as buffer in the medium (Table V)* — The dried and the de-fatted dried



meats were used in 10 per cent and the dried bacto-beef in 2 per cent amounts. The de-fatted dried meat was prepared by extracting the dried meat with ether and alcohol according to the method employed for extracting beef heart for Kahn antigen (Kahn, 1928). A highly potent toxin was obtained by using dried meat as buffer. The hæmolysin titre ran parallel with the opacity titre and showed a fairly regular relationship to the mouse mld, but the lytic effect on sheep's red-blood corpuscles which was fully visible when the tubes were incubated for two hours in the water-bath at 37°C, and then left in the cold room overnight, was more partial than complete giving an impression of disintegration rather than that of a true lytic phenomenon. By using de-fatted dried meat the hæmolysin content was increased fivefold, while the mouse mld remained at the same level. This hæmolysin differed from the one described above in its lytic effect which was visible within a few minutes and was practically complete within one hour at 37°C. Two per cent bacto-beef had the same effect as the de-fatted dried meat, but the mouse mld was only 50 per cent of what was obtained in the other two media. The above results, therefore, indicate that probably two kinds of hæmotoxins could be elaborated by *Clostridium perfringens* (type A) depending upon the medium used. In this series of experiments the potency of toxin was higher with NaCl than without it. For the purposes of brevity, in subsequent references in this paper, the hæmotoxin which reacted slowly will be called hæmotoxin I and the other which showed rapid lytic action will be called hæmotoxin II.

8 *Relationship of hæmolytic titre, opacity titre and mouse mld of Clostridium perfringens toxins produced in liver and veal digest medium (Table VI)*—Only those toxins whose titres had been determined to the end-point were taken into account. When 10 per cent dried meat was used as buffer in the liver and veal digest medium there was very little fluctuation in the relationship between mld and mod or mhd. The opacity titres maintained throughout a more constant ratio to mld than the hæmolytic titres, even in the presence of de-fatted dried meat or bacto-beef which produced hæmolysin of exceptionally high titre. The mean mld/mod and mld/mhd ratios were 1.476 and 1.465 respectively. The latter seemed to vary slightly with the conditions in the media and incubation period. The mod/mhd ratios varied between 0.75 and 1.25 with a mean reading of 1.02 and the tendency to discrepancy was more marked when the incubation period was extended beyond two to three days. With 10 per cent de-fatted dried meat or 2 per cent bacto-beef the mld/mhd ratio rose to 1.25, whereas that between mld and mod remained unchanged, indicating that the hæmotoxin elaborated in such media might be due to a different toxin other than the one elaborated by media containing dried meat described above. With de-fatted dried meat or bacto-beef the mod/mhd ratios were 1.5 and 1.10 respectively, the difference being presumably due to the actual differences in their mld titres.

9 *Lethal factor of hæmotoxin II (Table VII)*—Several toxins of variable strength containing one or both kinds of hæmotoxin (I and II) mentioned above were put up for neutralization test against the U.S. Standard Perfringens Antitoxin. The results showed that 0.2 unit of the antitoxin completely neutralized 20 mld

of toxin containing hæmotoxin I and only 10 m l d of toxin containing hæmotoxin II and possibly hæmotoxin I. It is probable that the extra amount of lethal effect shown by the latter was due to the presence of another toxin which contributed an additional lethal effect requiring for neutralization a proportionately larger amount of the standard antitoxin in which the anti-hæmotoxin II component was absent (Stewart, *loc cit*).

10 *Stability of perfringens toxins produced in liver and veal digest broth (Table VIII)*—It took about 40 days to de-toxify a toxin by means of 0.3 per cent formalin at 37°C. At the end of this period 0.1 cc of the undiluted toxoid gave negative opacity test but with 0.3 cc the reaction was still positive. An intraperitoneal injection of 1 cc of the material at this stage did not, however, kill mice (the original m l d being 0.01 cc). A toxoid could, therefore, be declared safe when 0.1 cc of the undiluted material gave a negative opacity reaction which actually proved much more sensitive than the lethal effect in mice for testing the loss of toxicity (Seal and Stewart, 1941).

The stability or rate of deterioration of several toxins of variable titres and hydrogen-ion concentrations was studied by keeping them at ordinary room temperature which varied between 82°F and 99°F during the observation period, and determining their toxicity at intervals by means of the opacity test, confirmed when necessary, by mouse test. The results showed that there was a 50 per cent loss in the potency of all toxins in the first 20 days and thereafter the rate of loss was considerably slower, there being only 10 per cent decrease in the next 30 days. It may be noted that the opacity titres maintained a constant ratio with m l d in spite of the deterioration indicating the intimate relationship between the two factors. It was further observed that the liquid toxin containing hæmotoxin II deteriorated at a much higher speed, a loss of 50 per cent of the potency was recorded in five days even when the toxin was preserved in the cold room. The pH of all the toxins tested remained throughout in the neighbourhood of 6.0. Although all of them showed a heavy loss in potency they still remained sufficiently active after 50 days at ordinary room temperature to serve as fairly good toxins.

## DISCUSSION

Several preliminary observations have been made in the present studies on the production of toxin of *Clostridium perfringens* (type A) in liver and veal digest broth modified in various ways yielding results which may be of interest to the future studies of the problem.

This digest broth afforded a good basic medium for producing a highly potent perfringens toxin at a comparatively low cost as the addition of expensive commercial peptone had been eliminated. The expense was further reduced by the use of dried meat (usually thrown away as waste) in place of fresh chopped meat as buffering agent. Of the various combinations tried the best result was obtained with the digest broth containing 0.25 per cent glucose, 10 per cent dried meat and 0.4 to 0.5 per cent sodium chloride at pH 7.8. The optimum period of

incubation was between 18 and 20 hours at 37°C. The medium showed further improvement when the ordinary dried meat was replaced by de-fatted dried meat. In the former case toxin corresponding to the zeta type and in the latter a combination of both zeta-toxin and alpha-lysin of Prigge (1936) were obtained. The inclusion of both the types has been advocated by several workers for the production of antitoxin. Perhaps the medium could be still further improved by taking better care in using properly de-fatted meat for digestion.

From the evidence obtained in the present studies there were possibly two antigens to constitute a more complete *Clostridium perfringens* (type A) toxin. antigen I corresponded to zeta-toxin of Prigge and contained lethal, hæmolytic opacity and possibly necrotic\* factors, all of which might be assumed on the basis of the results obtained to be due to one and the same antigen. For, in spite of the variation in the potency of toxin caused by certain modifications such as absence of glucose, withdrawal of meat, varying the incubation period etc., both the opacity and hæmolytic titres maintained more or less a constant ratio of approximately 1:5 with the lethal factor. In the process of deterioration, also, the same ratio was maintained throughout the entire period of observation. The hæmolytic factor in this case, however, was fully manifest only when the test was carried out by incubating the hæmolytic mixture for two hours in a water-bath at 37°C followed by overnight refrigeration. The reaction appeared more like a toxic disintegration than a true lytic phenomenon.

Antigen II corresponded to the alpha-lysin of Prigge and to hæmolysin A of Schnayerson and Samuel (1930) and was predominantly a hæmolytic factor. This factor, as was also shown by Stewart (*loc cit*), could not be produced in media containing fat or lipid substances and hence it was absent in the toxin obtained in liver and veal digest broth containing fresh or dried meat and appeared in high potency in conjunction with the other factors when the dried meat was extracted with ether and alcohol before being added to the broth. A distinct hæmolysis in the form of true lysis was obtained with a 1:2,500 dilution of the liquid toxin and the reaction was complete within one hour at 37°C. The results of neutralization test with the U. S. Standard *Perfringens* Antitoxin showed that this antigen also possessed a lethal factor for mice. Stewart was successful in isolating it from other antigens by passing the toxin through graded collodion membranes.

From the studies on the rate of deterioration of antigen I (zeta-toxin) in the liquid state at room temperature varying between 82°F and 99°F over a period of 50 days it was observed that although the deterioration was somewhat rapid within the first 20 days the residual toxin remained sufficiently active to serve as a fairly good toxin. Antigen II, on the other hand, was much more unstable, it deteriorated at a much higher speed, a loss of 50 per cent of the potency being recorded in five days' time even when it was preserved in the cold.

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\* No attempt was made to study the necrotic factor as it had been shown by others to run parallel with the lethal factor.

The existence of two different antigens in a more complete perfringens toxin, as suggested by the results noted above, should, however, be finally established by their complete isolation. Since antigen II was not only highly hæmolytic but, to a certain extent, was also lethal to mice, further investigations of more intensive nature in animals should be undertaken to evaluate the pathogenesis and antigenic capacity of this antigen, although some of the workers, namely Schnayerson and Samuel (*loc cit*) and Prigge (*loc cit*), on the basis of certain animal experiments, favoured the supposition that hæmotoxin II was less important than hæmotoxin I in the ætiology of gas gangrene. It may also be worth while to consider the inclusion of both the antigens in the production of antitoxin pending further information concerning *in vivo* action of antigen II. For testing such antitoxins a toxin containing both of them and the test dose with a stated number of mld of hæmotoxin II would be necessary.

#### SUMMARY

1 A modified liver and veal digest broth without commercial peptone for the production of a very potent *Clostridium perfringens* (type A) toxin has been described.

2 Studies of the lethal hæmolytic and opacity factors contained in the toxin indicate that possibly a more complete perfringens toxin (type A) is essentially composed of two antigens, each of which is characterized by a different type of hæmotoxin.

3 Further work on this subject, particularly for the production and standardization of the toxin and antitoxin, has been suggested.

#### ACKNOWLEDGMENTS

My grateful thanks are due to Doctors Sarah E Stewart and Ida A Bengtson of the National Institute of Health, Washington, D C, for their kind help, and to the authorities and staff of this Institute for affording all necessary facilities to carry out this work.

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TABLE I.

*Effect of various substances added to liver and veal digest broth at pH 7.8 and incubated at 37°C for 18 hours, on the production of Clostridium perfringens toxin*

Medium number	Meat, per cent	CaCO <sub>3</sub> , per cent	Sodium thio gly collate, per cent	Glucose, per cent	NaCl, per cent	Final pH	Mouse m l.d., c.c.	M o d., c.c.	M h d., c.c.
1	Dried 10.0	Nil	Nil	0.1	Nil	6.5	0.01	0.002	0.0025
2	"	"	"	0.25	"	6.2	0.006	0.0015	0.0015
3	"	"	"	0.5	"	5.8	0.008	0.0015	0.002
4	"	"	0.05	0.1	"	6.5	0.024	0.006	0.006
5	"	"	0.05	0.25	"	6.2	0.032	0.006	0.008
6	"	"	0.05	0.5	"	5.6	0.025	0.006	0.006
7	Nil	"	0.05	0.1	"	6.0	<0.05>0.025	0.01	0.01
8	"	"	0.05	0.25	"	5.4	<0.1>0.05	>0.01	>0.01
9	"	"	0.05	0.5	"	5.2	>0.1	>0.01	>0.01
10	"	2.0	Nil	0.1	"	6.4	<0.05>0.025	0.01	0.01
11	"	2.0	"	0.25	"	6.2	<0.1>0.05	>0.01	>0.01
12	"	2.0	"	0.5	"	6.0	<0.1>0.05	>0.01	>0.01
13	"	Nil	"	0.1	"	6.0	<0.1>0.05	>0.01	>0.01
14	"	"	"	0.25	"	5.3	>0.1	>0.01	>0.01
15	"	"	"	0.5	"	4.8	>0.1	>0.01	>0.01
16	Dried 10.0	"	"	0.1	0.5	6.4	<0.02>0.01	0.0025	0.003
17	"	"	"	0.25	0.5	6.1	<0.01>0.005	0.002	0.002
18	"	"	"	0.5	0.5	5.8	<0.02>0.01	0.0025	0.003
16a*	"	"	"	0.1	Nil	6.4	<0.02>0.01	0.0025	0.003
17a*	"	"	"	0.25	"	6.2	<0.01>0.005	0.002	0.002
18a*	"	"	"	0.5	"	5.8	<0.02>0.01	0.0025	0.003
19	Fresh beef 20.0	"	"	0.1	"	6.4	>0.02	0.005	0.005
20	"	"	"	0.25	"	6.1	<0.02>0.01	0.003	0.004
21	"	"	"	0.5	"	5.6	>0.02	0.006	0.006
22	"	"	"	0.1	0.5	6.5	<0.02>0.01	0.004	0.004
23	"	"	"	0.25	0.5	6.2	<0.02>0.01	0.003	0.003
24	"	"	"	0.5	0.5	5.8	>0.02	0.005	0.005

\* Composition same as media Nos 1, 2 and 3 respectively

TABLE II

*Showing the comparative efficiency of liver and veal digest broth and peptone beef infusion broth at pH 7.8 and incubated for 16 hours in the production of perfringens toxin*

Medium number	Medium base	Meat, per cent	Glucose, 0.25 per cent	NaCl, 0.5 per cent	Final pH	Mouse m.l.d., cc	M.o.d., cc	M.h.d., cc
33	Liver and veal digest broth	Dried 10.0	+	0	6.3	<0.01 >0.005	0.002	0.002
34	"		+	+	6.1	0.0075	0.0015	0.0015
35	Peptone beef infusion		+	0	6.6	0.02	0.004	0.004
36	"	"	+	+	6.6	0.01	0.003	0.003
SR12 (6/1)	,	Fresh 20.0	+	+	6.2	0.02	0.004	0.005

TABLE III

*Effect of varying the incubation period on the production of toxin in liver and veal digest medium at pH 7.8 with or without dried meat*

Medium number	Glucose, 0.25 per cent	Dried meat 10 per cent	NaCl, 0.5 per cent	Incubation period, hours	Final pH	Mouse m.l.d., cc	M.o.d., cc	M.h.d., cc
25	+	—	—	6	5.4	<0.1 >0.05	0.02	0.02
26	+	—	—	9	5.0	<0.05 >0.025	0.01	0.015
27	+	—	—	12	4.8	<0.1 >0.05	0.015	0.02
28	+	+	—	6	6.0	0.02	0.004	0.005
29	+	+	—	9	6.0	0.015	0.003	0.004
30	+	+	—	12	6.1	0.0125	0.002	0.003

TABLE IV

*Effect of varying the incubation period on the production of Clostridium perfringens toxin in liver and veal digest medium with 10 per cent dried meat, initial pH 7.8, and with or without glucose*

WITH 0.25 PER CENT GLUCOSE							WITHOUT GLUCOSE						
Medium number	Incubation period	Final pH	Mouse mld, cc	M o d, cc	M h d, cc	M l d per cc	Medium number	Incubation period	Final pH	Mouse mld, cc	M o d, cc	M h d, cc	M l d per cc
28	6 hours	6.0	0.02	0.004	0.005	50	58	6 hours	6.8	0.025	0.005	0.004	40
29	9 "	6.0	0.015	0.003	0.004	66	59	9 "	6.8	0.02	0.004	0.002	50
30	12 "	6.1	0.0125	0.002	0.003	80	60	12 "	6.6	<0.02>0.01	0.004	0.003	60 (approx.)
17a	16 "	6.2	<0.01>0.005	0.0015	0.002	133	61	16 "	6.6	<0.02>0.01	0.004	0.004	66 "
43	20 "	6.2	0.006	0.0015	0.0015	166	54	20 "	6.1	0.0125	0.0025	0.0025	80
46	24 "	6.2	<0.01>0.005	0.002	0.0025	133	53	24 "	6.2	0.01	0.0025	0.0025	100
45	36 "	6.0	0.01	0.002	0.0025	100	52	36 "	6.2	0.015	0.003	0.003	66
51	2 days	6.3	0.0125	0.003	0.003	80	42	2 days	6.2	0.02	0.005	0.007	50
50	3 "	6.4	0.025	0.005	0.007	40	41	3 "	6.4	0.05	>0.01	>0.01	20
40	4 "	6.4	0.05	0.01	0.01	20	40	4 "	6.6	>0.05<0.1	>0.01	>0.01	10 (approx.)
48	5 "	6.5	>0.1	0.02	0.02	<10	39	5 "	6.6	>0.1	>0.01	>0.01	<10
47	6 "	6.5	>0.1	0.05	0.04	<10	38	6 "	6.4	>0.1	>0.01	>0.01	<10
							37	7 "	6.4	>0.1	>0.01	>0.01	<10

TABLE V

*Effect of dried meat, de-fatted dried meat, dried bacto-beef and NaCl on the production of perfringens toxin in liver and veal digest medium at pH 7.8, incubated 18 hours*

Medium number	Meat, per cent	Glucose, per cent	NaCl, per cent	Final pH	Mouse m l d, c o	M o d, c o	M H D (SHEEP'S RED BLOOD CELLS), c o	
							1 hour at 37°C	Overnight
55	Dried 10 0	0 25	0 5	6 0	<0 01>0 005	0 002		0 002
56	De fatted dried 10 0	0 25	0 5	6 5	<0 01>0 005	0 002	0 0006	0 0004
57	Dried bacto beef 2 0	0 25	0 5	6 5	<0 02>0 01	0 004	0 0006	0 0001
62	Dried 10 0	0 25	0 4	6 2	0 01	0 0025		0 002
63	"	0 25	0	6 2	<0 02>0 01	0 003		0 0025
64	De fatted dried 10 0	0 25	0 4	6 2	<0 01>0 005	0 002	0 0004	0 0004
65	"	0 25	0	6 2	0 02	0 004	0 002	0 002



TABLE VI

*Relationship of hemolytic titre, opacity titre, and mouse mld of Clostridium perfringens toxins produced in liver and veal digest media enriched by various substances*

Medium number	Meat, per cent	Glucose, per cent	Sodium thioglycollate, per cent	NaCl, per cent	Incubation period	Mouse mld, cc	Vod, cc	Mhd, cc	$\frac{Mld}{Mod}$	$\frac{Mld}{Mhd}$	$\frac{Mod}{Mhd}$
1	Dried 10.0	0.1	Nil	Nil	16-18 hours	0.01	0.002	0.0025	5	1	0.8
2	"	0.25	"	"	"	0.006	0.0015	0.0015	4	4	1.0
3	"	0.5	"	"	"	0.008	0.0015	0.002	5.3	4	0.75
4	"	0.1	0.05	"	"	0.024	0.006	0.006	4	4	1.0
5	"	0.25	0.05	"	"	0.032	0.006	0.008	5.3	4	0.75
6	"	0.5	0.05	"	"	0.025	0.006	0.006	4.1	1.1	1.0
33	"	0.25	Nil	"	"	0.01	0.002	0.002	5	5	1.0
34	"	0.25	"	"	"	0.0075	0.0015	0.0015	5	5	1.0
55	"	0.25	"	0.5	"	0.01	0.002	0.002	5	5	1.0
62	"	0.25	"	0.5	"	0.01	0.0025	0.002	4	5	1.25
28	"	0.25	"	0.5	6 hours	0.02	0.004	0.005	5	4	0.8
29	"	0.25	"	0.5	9 "	0.015	0.003	0.004	5	3.75	0.75
30	"	0.25	"	0.5	12 "	0.0125	0.002	0.003	0.2	4.1	0.66
11	"	0.25	"	0.5	20	0.006	0.0015	0.0015	4	4	1.0

15		0.25	,	0.5	36	0.01	0.002	0.0025	5	4	0.8
51	,	0.25	"	0.5	2 days	0.0125	0.003	0.003	4.1	4.1	1.0
50	,	0.25	"	0.5	3 "	0.025	0.005	0.007	5	3.5	0.71
49	"	0.25	"	0.5	4 "	0.05	0.01	0.01	5	5	1.0
58	,	Nil	"	0.5	6 hours	0.025	0.005	0.004	5	6	1.25
59	"	"	"	0.5	9 "	0.02	0.004	0.002	5	10	2.0
60	"	"	"	0.5	12 "	0.02	0.004	0.003	5	6.6	1.3
61	"	"	"	0.5	16 "	0.02	0.004	0.004	5	5	1.0
54	"	"	"	0.5	20 "	0.0125	0.0025	0.0025	5	5	1.0
57	"	"	"	0.5	24 "	0.01	0.0025	0.0025	4	4	1.0
52	"	"	"	0.5	36 "	0.015	0.003	0.003	5	5	1.0
42	"	"	"	0.5	2 days	0.02	0.005	0.007	4	3	0.7

56	De-fatted dried 100	0.25	Nil	0.5	18 hours	0.01	0.002	0.0004*	5	25	5
57	Bacto beef 20	0.25	"	0.5	18 "	0.02	0.004	0.0004*	5	25	10

\* M h d of hemotoxin II

TABLE VII

Results of neutralization test of different toxins against 0.2 unit of the Standard Perfringens Antitoxin

Toxin lot number	Liver and veal digest medium	Hæmotoxin present	M l d, c c	NUMBER OF M L D AGAINST 0.2 UNIT OF THE STANDARD ANTITOXIN			
				10	20	30	40
2	With dried meat	I	0.006	Complete protection	Complete protection	No protection	No protection
26	"	I	0.02	"	"	"	"
34	"	I	0.01	"	"	"	"
57	With bacto beef	I and II	0.02	"	No protection	"	"
64	With de-fatted dried meat	I and II	0.01	"	"	"	"

TABLE VIII

*Results of incubating the toxins produced in liver and veal digest media at room temperature (82°-99° F) for 50 days*

Medium number	Liver and veal digest medium	Final pH	ORIGINAL TITRES		OPAITY TITRES (00) AT ROOM TEMPERATURE AFTER			PERCENTAGE OF POTENCY LOST	
			M.I.d., 00	M.O.d., 00	20 days	40 days	50 days	M.I.d (00) after 50 days	
1	With dried meat and glu cose	6.5	0.01	0.002	0.004	0.005	0.006	0.03	50
2	"	6.2	0.006	0.0015	0.003	0.0033	0.004	0.02	50
3	"	5.8	0.008	0.0015	0.003	0.0033	0.004	0.02	50
17	per cent NaCl + 0.5	6.1	0.01	0.002	0.004	0.005	0.005	0.025	50



## ON THE COMPLEMENT AND ESTERASE ACTIVITY OF SERA OF SOME COMMON LABORATORY ANIMALS

BY

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[Received for publication, December 31, 1941]

THE true nature of serum complement is unknown in spite of many investigations on the subject. In many respects its action resembles that of a ferment. The curves of complement activity are like those of enzyme action (Thiele and Embleton, 1914-15) and most evidence supports the ferment hypothesis of the nature of complement. As to the nature of the ferment, again there are divergent views. Though the results obtained by Dick (1913) would seem to indicate that hæmolytic complement is of the nature of a proteolytic enzyme, the majority of workers on the subject consider that its action is similar to that of a lipase. The presence of a true fat-splitting enzyme in the sera of animals has not been established, but there is strong evidence of the presence of an esterase (i.e. an enzyme capable of hydrolysing the esters of the lower fatty acids) in varying amounts.

It is believed that the function of these esterases is to hydrolyse the lower fatty esters in the blood prior to their oxidation, and in this respect they resemble the esterases present in the tissues generally and have no specific function. Several workers have attempted to identify serum complement with serum esterase. According to Jobling and Bull (1913) there is parallelism between the esterase content and the complement strength of the sera of different animals. The work of Gordon and Wormall (1929) shows that, whilst the esterase and complementary activity of the serum tend to run parallel, there are occasional discrepancies which do not support the view that the action of complement is a simple esterase function. For instance, guinea-pig serum when heated to 56°C for 30 minutes loses all detectable complement action but still retains a distinct esterase activity. Our observations on rabbits' heated serum also support this finding. There are

other important findings which tend to show that the action of the serum complement is not due to the activity of a lipase system. A comparison of the lipolytic activities of the protein fractions of serum with those of the original serum shows that these protein preparations possess a high percentage of the lipolytic power of the original serum but have no hæmolytic action on sensitized red blood cells (Dawson and Platt, 1928). Again, serum inactivated either by zymine or by ammonia has esterase activity equal to that of the original serum but no hæmolytic complement action. The results so far obtained therefore lead to no definite conclusions as to the nature of the complement action.

### EXPERIMENTAL

A comparison was made of the esterase and complement activity of the sera of different animal species. The esterase activity was determined according to the technique described earlier (Roy, 1938). Various dilutions of the serum were allowed to act upon ethyl butyrate in the presence of a phosphate buffer solution of pH 7.6. Suitable controls for the ester and the serum were run at the same time. The flasks were incubated for 21 hours at 37°C with toluene as preservative and titrated with N/50 NaOH, phenolphthalein being used as indicator. The complement was titrated against sheep's r b c, sensitized with anti-sheep rabbitamboceptor. 0.3 c.c. of sheep's r b c and 0.1 c.c. of each dilution of the complement were used, the total volume of 1 c.c. being made up with the addition of 0.6 c.c. of sterile normal saline. To ascertain the effect of natural hæmolysins, if any, control experiments with non-sensitized sheep's r b c were run in most of the cases. Diluted guinea-pig complement 5 m h d was used as control.

The results given in the tables are typical of a number of experiments done on the sera of each species of animals.

The data given reveal some interesting facts. It will be seen that the sera of the different species of animals investigated may be grouped under four distinct heads according to their relative esterase and complement activity —

*Group I* —Sera having little or no esterase activity but moderate complement activity, e.g. human beings, dog, monkey (*Rhesus*). The sera of these species are characterized by the fact that they all possess fairly active natural hæmolysins capable of lysing even non-sensitized sheep's r b c. This is a corroboration of the results reported by Kolmer (1923).

*Group II* —Sera having neither appreciable esterase nor complement activity, e.g. ox, sheep, buffalo and goat. The sera of the ox and buffalo, however, produce strong agglutination of sheep's r b c.

*Group III* —Sera showing appreciable esterase activity but little or no complement action, e.g. cat and horse. Horse serum was found to have no complement action in sheep's r b c, even when used undiluted.

*Group IV* —Sera showing both strong esterase and strong complement activity, e g rabbit and guinea-pig

It is apparent that the blood sera of other animals must fall into one or other of these groups

#### SUMMARY AND CONCLUSIONS

1 The esterase and the complement activity of the blood sera of animals vary within wide limits

2 The common laboratory animals may be grouped under four distinct heads according to the relative esterase and complement activity of their blood sera

3 Except in the case of animals coming under groups II and IV, these two properties do not run parallel

4 That esterase activity and complement activity are not identical is apparent from groups I and III, where negative or very low esterase content is associated with high complement action and vice versa

#### ACKNOWLEDGMENT

My grateful thanks are due to the Imperial Serological Department, Calcutta, for the regular supply of sensitized cells and guinea-pig complement

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TABLE I

Group I.

Nature of serum	Dilution of complement, etc	Esterase activity in terms of N/50 NaOH, c c	HÆMOLYSIS							
			SENSITIZED R B C				NON SENSITIZED R B C			
			1/4 hour	1 hour	2 hours	21 hours	1/4 hour	1 hour	2 hours	21 hours
Human	Ester control	0.6	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
	Serum control (dilution 1/2)	0.4	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
	Serum 1/2	0.9	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
	" 1/4	0.8	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
	" 1/8	0.6	90 per cent	95 per cent	98 per cent	98 per cent	80 per cent	90 per cent	95 per cent	98 per cent
	" 1/16	0.6	—	2 per cent	5 per cent	20 per cent	—	—	—	—
	Guinea-pig complement 5 m h d 1/50	7.3	+++++	+++++	+++++	+++++	—	—	—	—

Monkey	Water control	06	+++++	+++++	+++++	+++++	+++++	+++++	98 per cent	+++++	+++++
	Serum control (dilution $\frac{1}{2}$ )	04	+++++	+++++	+++++	+++++	+++++	+++++	80 per cent	+++++	90 per cent
	Serum 1	17	+++++	+++++	+++++	+++++	+++++	+++++	50 per cent	+++++	5 per cent
	" 1	11	+++++	+++++	+++++	+++++	+++++	+++++	—	—	—
	" 1	08	—	—	—	—	—	—	—	—	—
Guinea pig complement 5 m h d 1/50	" 1	07	—	—	—	—	—	—	—	—	—
	" 1	07	—	—	—	—	—	—	—	—	—
	Guinea pig complement 5 m h d 1/50	88	+++++	+++++	+++++	+++++	+++++	+++++	—	—	—

Dog	Water control	04	+++++	+++++	+++++	+++++	+++++	+++++	10 per cent	+++++	+++++
	Serum control (dilution $\frac{1}{2}$ )	03	+++++	+++++	+++++	+++++	+++++	+++++	90 per cent	+++++	98 per cent
	Serum 1	07	+++++	+++++	+++++	+++++	+++++	+++++	50 per cent	+++++	5 per cent
	" 1	05	+++++	+++++	+++++	+++++	+++++	+++++	—	—	—
	" 1	05	—	—	—	—	—	—	—	—	—
Guinea pig complement 5 m h d 1/50	" 1	05	—	—	—	—	—	—	—	—	—
	" 1	05	—	—	—	—	—	—	—	—	—
	Guinea pig complement 5 m h d 1/50	53	+++++	+++++	+++++	+++++	+++++	+++++	—	—	—

+++++ = Complete hemolysis.

— = No hemolysis

TABLE II  
Group II.

Nature of serum	Dilution of complement, etc	Esterase activity in terms of N/50 NaOH, cc	HÆMOLYSIS							
			SENSITIZED R B C				NON SENSITIZED R B C			
			1/4 hour	1 hour	2 hours	21 hours	1/4 hour	1 hour	2 hours	21 hours
Sheep	Ester control	0.7								
	Serum control (undiluted)	0.2								
	Serum undiluted	0.9	—	—	—	—	—	—	—	—
	" 1/2	0.9	—	—	—	—	—	—	—	—
	" 1/4	0.6	—	—	—	—	—	—	—	—
Goat	Serum control (undiluted)	0.4								
	Serum undiluted	0.9	—	—	—	—	—	—	—	—
	Serum 1/2	0.5	—	—	—	—	—	—	—	—
	" 1/4	0.6	—	—	—	—	—	—	—	—
	Guinea-pig complement 5 m h d 1/80	0.8	+++++	+++++	+++++	+++++	—	—	—	—



TABLE III

Group III

Nature of serum	Dilution of complement, etc	Esterase activity in terms of N/50 NaOH, c c	HÆMOLYSIS							
			SENSITIZED R B O				NON-SENSITIZED R B O			
			1 hour	1 hour	2 hours	21 hours	1 hour	1 hour	2 hours	21 hours
Cat	Ester control	0.5	—	—	A trace	10 per cent	—	—	—	—
	Serum control (dilution 1/2)	0.5	—	—	—	—	—	—	—	—
	Serum 1/2	5.2	—	—	—	—	—	—	—	—
	" 1	3.5	—	—	—	—	—	—	—	—
	" 1/2	2.0	—	—	—	—	—	—	—	—
	" 1/4	1.2	—	—	—	—	—	—	—	—
	" 1/8	0.8	—	—	—	—	—	—	—	—
Guinea-pig complement 5 m h d 1/90	" 1/2	0.5	—	—	—	—	—	—	—	—
	" 1/4	4.4	+++++	+++++	+++++	+++++	—	—	—	—





## HISTOPATHOLOGY OF MANTOUX REACTION AND ERYTHEMA NODOSUM

BY

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[Received for publication, December 2, 1941 ]

THREE tuberculous patients showing more or less the same degree and type of reaction to intradermal tuberculin test were chosen. 0.1 c.c. of 1 in 1,000 dilution of O.T. was given intradermally on the flexor aspect of the forearm to each of the patients. After 48 hours all the three developed erythema extending over an area of about 4 cm. in diameter, and induration of about 3 cm. Under block anaesthesia a portion of the skin and subcutaneous tissue was removed for pathological examination. Biopsy material was obtained from each of the three patients on the 2nd, 5th and 15th day after injection respectively. Histological appearances were as follows —

*Case 1 — 2nd day (Plate II, figs 1 and 2)* — Skin shows very little abnormality. Dermal papillae are normal. Deeper down there are perivascular inflammatory foci extending down to the sebaceous glands and sweat glands and even the subcutaneous fat. The capillaries are all engorged and show swelling of the endothelium. There is beginning of a thrombus in the lumen. The cells themselves are mostly polymorphs. Some of them are mononuclears. There are also numerous lymphocytes. There are no large foci of necrosis.

*Case 2 — 5 days (Plate II fig 3)* — Section shows slight hyperkeratosis of the surface epithelium with commencing desquamation. In the dermis there are small capillary vessels showing perivascular cuffs of inflammatory cells. In places the inflammatory reaction is more diffuse and extends deeper down in the dermis. The cells are mostly mononuclear histiocytes with here and there lymphoid cells. There is also proliferation of capillary endothelium as well as slight increase of fibroblasts. No necrotic foci are visible. In places the reaction can be seen round hair follicles and sweat glands.

*Case 3 — 15 days (Plate III, fig 4)* — Under the skin there are small foci of chronic inflammation most marked around the sweat glands. Well-marked







FIG 1  
Case 1 — Showing the inflammatory reaction extending down to the sebaceous gland H and L ( $\times 80$ )



FIG 2  
Case 1 — Showing the marked pericapillary infiltration and damage to the capillary endothelium H and E ( $\times 120$ )



FIG 3  
Case 2 — Showing slight hyperkeratosis and perivascular inflammatory cuffing of the dermis H and F ( $\times 80$ )

perivascular reaction and proliferation of the endothelium of the blood vessels are seen. The cells are mostly lymphocytes and histiocytes, with an occasional polymorphonuclear and eosinophile cells. The subcutaneous fat is also infiltrated. The surface epithelium shows slight desquamation.

*Discussion* — That the tuberculin reaction is not like ordinary inflammatory reaction is shown by the fact that there is evidence of damage to the endothelium of blood vessels which show perivascular cuffing and proliferation of the endothelium with occasional thrombus formation. At the beginning polymorphonuclear cells seem to predominate. Later on lymphoid cells and histiocytes appear. Fibroblastic proliferation is also evident in the later stages. The histological appearances, however, do not substantiate the claim of Kitamura and Tasaki (1934) that in the tuberculin reaction a tubercular structure is always formed in the cutis or subcutis.

### *Erythema nodosum*

Biopsy materials for the study of erythema nodosum were taken from two patients who developed the specific nodules few days after Mantoux test was done.

*Case 1 (Plate III, figs 5 and 6)* — The patient, Hindu male, 17 years, sought treatment for irregular fever and dry cough of a month's duration. He was diagnosed as a case of tuberculosis of the hilum glands from clinical and radiological signs pertaining to enlarged hilum. The sputum which the patient spat out after administration of potassium iodide was repeatedly negative for T B. Stomach wash for examination was not done as it was thought unnecessary. His sister was an open case of pulmonary tuberculosis.

Report on his blood was as follows — \

R b c	4 75 mill
W b c	8,200
Hb, per cent	85

<i>Schilling's count</i>	Myelocytes	<i>Nil</i>
	Juveniles	$\frac{1}{2}$ per cent.
	Stabs	19 "
	Segmentars	67 "
	L Mono	5 ,
	Lympho	6 "
	Eosinophile	2 5 "

Sedimentation rate	32
W R	Negative

Mantoux test was done on admission with 0.1 c.c. of 1 in 1,000 dilution. It produced a very severe local reaction leading on to actual ulceration. Four days after the test was done, nodules, numerous over both shins, and a few on the dorsal aspect of the forearm, appeared. One of the nodules was excised under local anaesthesia on the second day after the appearance of the nodules. The histological appearance is given in Plate III, figs 5 and 6.

## AN IMPROVED METHOD FOR THE ESTIMATION OF VITAMIN B<sub>1</sub> IN FOODS BY THE THIOCHROME REACTION

BY

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[Received for publication, December 31, 1941]

THE thiochrome test for vitamin B<sub>1</sub> was first described by Jansen (1936). The principle of the method is that vitamin B<sub>1</sub> is oxidized into its fluorescent derivative thiochrome which is extracted with isobutyl alcohol and the fluorescence estimated in a fluorimeter. Since 1936 several groups of workers have attempted to perfect the method of assay, as applied to foodstuffs (Pyke, 1937, 1939, Hennessy and Cerecedo, 1939, Aykroyd, Krishnan, Passmore and Sundararajan, 1940, Houston, Kon and Thompson, 1940) and human urine (Westenbrink and Goudsmit, 1937, Karrer, 1937, Marrack and Hollering, 1939, Wang and Harris, 1939, Hills, 1939, Jowett, 1940). Though some success has been achieved in improving the method as applied to urine, difficulties have been encountered in evolving a simple general procedure for application to all kinds of food materials. For example, Aykroyd *et al* (*loc cit*) found their method applicable to cereals but not to pulses, yeast, etc. Hennessy and Cerecedo (*loc cit*) have also stated that the assay of the vitamin directly in food extracts was not possible with certain types of foods, though no unusual interfering fluorescent materials are met with. For dealing with such foods, they proposed adsorbing the vitamin on synthetic zeolite. Pyke (1939) proposed a simple method of estimating the vitamin in foodstuffs, in which the intensity of fluorescence due to thiochrome was estimated visually. In attempting to follow this method the author found that the visual comparison of solutions containing small amounts of thiochrome, in the presence of other fluorescent materials, was only roughly quantitative, being influenced by the personal factor. Erroneous results were also obtained when the fluorescence was estimated in a fluorimeter.

It is thus evident that there is need for a simple and reliable method of estimating vitamin B<sub>1</sub> in foods. The present paper describes a technique, based on the thiochrome method, which appears to be satisfactory.



*Apparatus*—The fluorimeter used was described in a previous paper (Swaminathan, 1942)

Before describing the final procedure in detail, various preliminary investigations carried out with the object of discovering a satisfactory technique will be considered —

(1) *Preliminary adsorption*—A few experiments were carried out in which the vitamin was adsorbed on Fuller's earth from unpurified extracts of foods. It was found that in the case of some foods, e.g. pulses and yeast, adsorption was incomplete and variable. After precipitation of crude extracts of yeast by basic lead acetate following the hydrolysis of cocarboxylase to the free vitamin, adsorption of the vitamin was complete. Recovery values were of the same order as those obtained by the method described in this paper. Preliminary adsorption was accordingly omitted. It is possible that with certain types of foods the adsorption technique might give slightly better recoveries than the direct method, if applied to purified extracts.

(2) *Removal of colouring and interfering fluorescent and reducing substances by treatment with basic lead acetate*—Most of the foods examined contained substances of this nature. Treatment of the extracts with basic lead acetate removed the greater part of them. The results obtained with a few foods before and after treatment with lead acetate are given in Table I —

TABLE I  
*Results of preliminary experiments*

Source of extract.	* Method of treatment	Reading due to 'blank' test' (scale divisions)	Vitamin B <sub>1</sub> found, µg/g	RECOVERY IN PER CENT OF VITAMIN B <sub>1</sub> ADDED TO		Recovery in per cent of thiochrome added to final isobutyl alcohol extracts	
				Food.	Extract just before oxidation		
Cambu ( <i>Pennisetum typhoides</i> )	1	15	24	3.0	50	50	60
	2	12	26	3.8	60	62	73
	3	9	24	3.8	60	60	75
Yeast dried ( <i>Torula</i> strain)	1	12	29	25.0	52	55	73
	2	8	27	26.4	56	58	75
	3	6	27	26.1	63	67	84

- \* 1 The original extract obtained after treatment with pepun or taka-diastase, adjusted to pH 4 was used for oxidation
- 2 Extract (1) purified by treatment with basic lead acetate, with excess of lead removed, the pH adjusted to 4.
- 3 Extract (2) after being washed once with isobutyl alcohol.

TABLE I—concl'd

Source of extract	* Method of treatment	Reading due to 'blank'—'test' (scale divisions)		Vitamin B <sub>1</sub> found, µg/g	RECOVERY IN PER CENT OF VITAMIN B <sub>1</sub> ADDED TO		Recovery in per cent of thiochrome added to final isobutyl alcohol extracts
					Food	Extract just before oxidation	
Cabbage ( <i>Brassica oleracea capitata</i> )	1	16	16	Nil	45	50	72
	2	3	8	0.7	60	65	82
	3	3	8	0.7	65	68	85
Carrot ( <i>Daucus carota</i> )	1	14	14	Nil	47	60	68
	2	4	7	0.4	55	60	78
	3	4	7	0.4	60	64	85

\* 1 The original extract obtained after treatment with pepsin or taka diastase, adjusted to pH 4, was used for oxidation

2 Extract (1) purified by treatment with basic lead acetate, with excess of lead removed, the pH adjusted to 4

3 Extract (2) after being washed once with isobutyl alcohol

It will be seen that this treatment reduced the value of the 'blank' and increased the percentage recovery of the added vitamin, besides removing a large proportion of the colouring matter and reducing interfering substances. Further, with two vegetables, e.g. cabbage and carrots, no value for vitamin B<sub>1</sub> content could be obtained before lead acetate treatment. This was evidently due to the fact that the increase in intensity of the fluorescence due to the thiochrome formed is offset by the reduction of part of the high 'blank' fluorescence during oxidation.

(3) *Preliminary washing with isobutyl alcohol*—Preliminary washing of the extracts obtained after lead acetate treatment with isobutanol reduced the value of the 'blank' and increased the recovery of vitamin (Table I). Harris (*loc cit*) also found that preliminary washing of urine with isobutyl alcohol significantly improved the accuracy of the results.

(4) *Recovery of added vitamin*—Many workers have reported the recovery of added vitamin ranging from 40 to 80 per cent, while others have reported values ranging from 66 to 85 per cent. In the present investigation, the recovery of added vitamin was 72 to 85 per cent with different methods. Westernbrink and Goud have used the method of Jovett and Goud, and the recovery was 68 per cent, while Aykroyd *et al* (loc cit) reported a recovery of 75 to 80 per cent. In the present investigation, the recovery of added vitamin was 72 to 85 per cent with different methods. Westernbrink and Goud have used the method of Jovett and Goud, and the recovery was 68 per cent, while Aykroyd *et al* (loc cit) reported a recovery of 75 to 80 per cent. In the present investigation, the recovery of added vitamin was 72 to 85 per cent with different methods.

urine inhibited the formation of thiochrome from added vitamin, while Marrack and Hollering (*loc cit*) reported that the final isobutanol 'test' solutions had an inhibiting effect on the fluorescence due to the added thiochrome. Jowett (*loc cit*) expressed the view that incomplete recovery of added vitamin was apparently due to losses occurring during the process of elution and oxidation.

To find out the causes for the loss of added vitamin and the stage at which the loss occurred, known amounts of the vitamin or thiochrome were added to the test materials at three different stages of the test as follows: (a) to the foodstuff at the beginning of the experiment and (b) to the purified extracts just before oxidation, known amounts of thiochrome in isobutanol solution were added (c) to the final test isobutanol solutions, to find the percentage inhibition caused by the unknown test solutions, on the fluorescence due to the added thiochrome. The thiochrome was prepared by oxidizing 40  $\mu$ g of the standard vitamin and extracting the thiochrome formed with 20 ml of isobutanol, so that 1 ml of the isobutanol extract represented the thiochrome obtained from 2  $\mu$ g of vitamin B<sub>1</sub>. Usually 1 ml of the thiochrome solution was added to discover the percentage inhibition, 1 ml of pure isobutanol being added to the control, the volume being thus kept the same in all the comparative tests. The percentage recovery was calculated by taking the intensity of fluorescence due to the same amount of thiochrome in pure isobutyl alcohol as 100 per cent. The results of the recovery experiments are given in Table I.

It will be seen that the values obtained for the recovery of the vitamin added to the food at the beginning of the experiment were approximately the same as those obtained in the case of the vitamin added to the purified extracts just before oxidation, ranging from 60 to 65 per cent. It is therefore probable that no appreciable loss of the vitamin occurs before the oxidation stage. On the other hand, the percentage recovery of the thiochrome added to the final 'test' isobutanol solutions was of a somewhat higher order, ranging from 75 to 85 per cent, indicating that 15 to 25 per cent of the losses can be ascribed to the inhibition caused by the unknown 'test' solution on the fluorescence due to thiochrome added or formed from the added vitamin. The results further show that there is some loss of the vitamin added to extracts, taking place during the process of oxidation and amounting to 15 to 25 per cent.

Incomplete recovery of added vitamin is therefore due to two factors: (a) the loss of the vitamin added to the food or food extracts occurring during the process of oxidation and (b) to the inhibiting effect of the unknown test solutions on the fluorescence due to the thiochrome formed from the added vitamin. Hence the practice of correcting the final vitamin values for the loss determined by recovery tests seems to be fully justified.

#### REGULATION OF THE QUANTITY OF FERRICYANIDE REQUIRED FOR OXIDATION

Several workers have shown that excess of ferricyanide lowers the reading given by the test by eliminating a part of the 'blank' fluorescence and probably



also by destroying part of the thiochrome formed (Hills, *loc cit*, Aykroyd *et al*, *loc cit*, Jowett, *loc cit*, Westenbrink and Goudsmit, *loc cit*) The amount of ferricyanide required for the maximum yield of thiochrome depends on the total oxidizable substances present besides vitamin B<sub>1</sub> and must be found by trial

It was observed in the present investigation that for a 12.6-ml quantity of purified food extracts, with or without added vitamin, the amount of 5 per cent ferricyanide required ranged from 0.3 ml to 0.5 ml. The relevant results are given in Table II —

TABLE II

*The effect of variation of the quantity of ferricyanide on the intensity of fluorescence*

Quantity of ferricyanide, ml	INTENSITY OF FLUORESCENCE (SCALE DIVISIONS)		
	Pure vitamin	Extract of lentil	Extract of yeast
0.0	6	12	12
0.1	48	26	27
0.2	46	32	30
0.3	42	32	35
0.4	40	31	34
0.5	39	28	30

## FINAL PROCEDURE

A For foodstuffs containing no cocarboxylase (cereals, pulses and vegetables)

*Extraction*—Ten to thirty grammes of the finely powdered or minced material containing approximately 10  $\mu$ g to 50  $\mu$ g of the vitamin, were placed in a conical flask. Ninety ml of water and 1 ml of 10 N H<sub>2</sub>SO<sub>4</sub> were then

added and the mixture heated in a water-bath at 40°C for 15 minutes. Pepsin, 0.5 g dissolved in 9 ml of water, was then added. To a second sample prepared as described above, 50 µg of standard vitamin B<sub>1</sub> were added to find out the percentage recovery. The mixtures were incubated at 38°C overnight. They were then heated in a water-bath maintained at 65°C to 70°C for 20 minutes, cooled and centrifuged.

*Removal of interfering substances with basic lead acetate*—To 60 ml of the clear extract, 12 ml of a N/1 solution of basic lead acetate were added, to precipitate the colouring matter and other interfering substances present. The precipitate was removed on the centrifuge. The excess of lead present was removed as lead sulphate by the addition of 2 ml of 10 N H<sub>2</sub>SO<sub>4</sub>. The clear solution was adjusted to pH 4 by the careful addition of 10 N NaOH.

*Preliminary washing with isobutyl alcohol*—Thirty ml of isobutyl alcohol were added to the extract obtained from above, the mixture shaken for 2 minutes and allowed to stand. The isobutyl alcohol layer was rejected. Aliquots of the aqueous extracts, usually 12.6 ml, corresponding to one-tenth of the original material taken for analysis, were used for estimation as described later.

#### B For foods containing cocarboxylase besides vitamin B<sub>1</sub> (yeast animal tissues, etc)

Kinnorsley and Peters (1938) have shown that in animal tissues and yeast vitamin B<sub>1</sub> is present mostly in the form of cocarboxylase, and that enzymatic hydrolysis of cocarboxylase to the free vitamin is essential before the thiochrome test can be applied. They have further shown that taka-diastase (containing taka-phosphatase) is capable of hydrolysing cocarboxylase quantitatively to vitamin B<sub>1</sub> in a short time. Taka-diastase was therefore employed in the present investigation when dealing with yeasts and animal tissues.

*Extraction*—A suitable quantity of the finely powdered or minced material (1 g to 2 g with yeast and 5 g to 10 g with animal tissue), containing approximately 10 µg to 50 µg of the vitamin, was suspended in 45 ml of N/10 H<sub>2</sub>SO<sub>4</sub>. The mixture was heated with constant stirring in a boiling water-bath for 20 minutes, allowed to cool and centrifuged. The residue was extracted once again in the above manner using 35 ml of N/10 H<sub>2</sub>SO<sub>4</sub>. In the case of extracts obtained from animal tissues, 8 ml of 25 per cent trichloroacetic acid containing 0.5 ml of 10 N NaOH were added to precipitate the protein derivatives present. The addition of trichloroacetic acid to yeast extracts is unnecessary.

*Hydrolysis of cocarboxylase to vitamin B<sub>1</sub>*—The pH of 4.0 required for enzymatic hydrolysis was attained by adding 2.3 ml of 4.8 N sodium acetate solution to the combined extracts. Taka-diastase (0.5 g) was added and the mixture thoroughly shaken. The solution was then made up to 100 ml by the addition of acetate buffer (pH 4), warmed to 38°C and incubated at 38°C for 5 to 6 hours.

The rest of the procedure, e.g. treatment with lead acetate and isobutyl alcohol, was the same as that followed for cereals and pulses

*Notes* —(1) The vitamin present in starchy foods, e.g. cereals and pulses, can be easily extracted by boiling N/10 H<sub>2</sub>SO<sub>4</sub>, but relatively large amounts of the solvent (200 ml. to 300 ml. for 10 g. of the material) are required to obtain anything like the complete extraction required for the procedure described

(2) Incubation of the extracts of cereals and pulses at pH 4 with taka diastase did not, as in the case of yeast and animal tissues, result in any increase in the values of vitamin B<sub>1</sub>. Hence the taka diastase treatment was omitted when dealing with cereals, pulses and vegetables

(3) Estimation of the vitamin B<sub>1</sub> content of a few samples of yeast and liver was carried out with and without preliminary digestion of the test material with pepsin. The results obtained were the same in both cases, so that preliminary digestion with pepsin appears to be unnecessary with yeast and animal tissues.

*The thiochrome test* —Aliquots of the extracts, usually 12.6 ml. of the purified extract equivalent to one-tenth of the original material taken, were measured into four glass-stoppered tubes (50 ml. capacity) marked B, T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> respectively. Standard vitamin B<sub>1</sub>, 5 µg., was taken in another tube marked S and the volume diluted to 12.6 ml. by the addition of water saturated with isobutyl alcohol. Two ml. of methyl alcohol were added to each. Two ml. of 40 per cent NaOH were then added to tube B containing the 'blank', followed by 15 ml. of isobutyl alcohol. The mixture was shaken for 2 minutes and allowed to stand. To tube S were added, in rapid succession, 0.2 ml. of 5 per cent ferricyanide, 2 ml. of 40 per cent NaOH, and 15 ml. of isobutyl alcohol. The mixture was well shaken for one minute and allowed to stand. The contents of tubes T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> were treated in the same manner as S, except that 0.3 ml., 0.4 ml. and 0.5 ml. of 5 per cent ferricyanide were added instead of 0.2 ml. Ten ml. of the clear filtered isobutyl alcohol extracts were used for the estimation of the amount of fluorescence present in the fluorimeter.

*Calculation* —The maximum reading obtained with any one of the three 'test' solutions T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub> was taken as the correct value. The value obtained for the 'blank' was allowed for in the usual way. The percentage recovery of added vitamin ranged from 60 to 80 with different foods. Hence all the values obtained for the test materials were corrected using the corresponding recovery values.

*Results* —The method described was applied to 19 foods, viz. 4 cereals, 6 pulses, 2 vegetables, 4 samples of yeast, ground-nut, rice polishings and sheep liver. The results are given in Table III. For the two samples of dried brewer's yeast, values of 34.0 µg. and 24.1 µg. per g. were obtained. Another sample of dried yeast (*Torula* strain) grown in the laboratory on a synthetic medium containing molasses gave a value of 26.1 µg., while a sample of dried distillery yeast gave the lower value of 12.9 µg. These figures appear to correspond well with those reported by Pavcek, Peterson and Elvehjem (1937) for yeasts grown on different media. Of the four cereals investigated, whole wheat contained 4.3 µg., raw milled rice 1.2 µg., raw husked rice 4.1 µg. and cambu 3.8 µg. These figures correspond closely with those reported by Aykroyd *et al.* (*loc. cit.*) for a large number of samples. All the six pulses investigated were found to be good sources of the vitamin, containing

2.1  $\mu\text{g}$  to 5.1  $\mu\text{g}$  Values of 7.4  $\mu\text{g}$  and 3.6  $\mu\text{g}$  were obtained for ground-nut and fresh sheep liver respectively Cabbage and carrot were found to be poor sources, containing 0.7  $\mu\text{g}$  and 0.4  $\mu\text{g}$  respectively On the whole the results obtained by the present method appear to correspond well with those reported by other workers using biological methods (Aykroyd *et al*, *loc cit*, Williams and Spies, 1938, Leong, 1940)

TABLE III

*Vitamin B<sub>1</sub> content of various foods*

Name of foodstuff	Botanical name	Vitamin B <sub>1</sub> , $\mu\text{g/g}$
<i>Cereals—</i>		
Cambu	<i>Pennisetum typhoideum</i>	3.8
Rice raw milled	<i>Oryza sativa</i>	1.2
Rice, raw husked	<i>Oryza sativa</i>	4.1
Wheat whole	<i>Triticum vulgare</i>	4.3
<i>Pulses—</i>		
Bengal gram	<i>Cicer arvense</i>	4.5
Black gram	<i>Phaseolus mungo</i>	4.1
Green gram	<i>Phaseolus radiatus</i>	4.5
Lentil	<i>Lens esculenta</i>	4.1
Red gram (1)	<i>Cajanus indicus</i>	3.8
Red gram (2)	<i>Cajanus indicus</i>	2.1
<i>Vegetables—</i>		
Cabbage	<i>Brassica oleracea capitata</i>	0.7
Carrot	<i>Daucus carota</i>	0.4
<i>Nuts—</i>		
Ground nut	<i>Arachis hypogea</i>	7.4
<i>Flesh foods—</i>		
Liver, sheep		3.6
<i>Yeasts—</i>		
Yeast, dried, brewer's (1)		34.0
Yeast, dried, brewer's (2)		24.1
Yeast, dried, <i>Torula</i> (grown on a synthetic medium containing molasses)		26.1
Yeast, dried, distillery		12.0

## DISCUSSION

The method described is rapid and simple It has been found to give reliable results with a wide range of materials, containing varying amounts of vitamin B<sub>1</sub> It was found possible with practice to assay six foods in two days The recovery of added vitamin is of the same order as that obtained with other thiochrome methods Further attempts to improve the recovery of added vitamin are being made

## SUMMARY

1 A simple method for the estimation of vitamin B<sub>1</sub> in foods, using the thiochrome reaction, has been described

2 Materials interfering in the thiochrome test were eliminated to a large extent by treatment with lead acetate and preliminary washing with isobutyl alcohol

3 Evidence has been produced to show that the incomplete recovery of added vitamin is due to (a) the loss occurring during the process of oxidation and (b) the inhibiting effect of unknown isobutyl alcohol 'test' solutions on the fluorescence due to the thiochrome formed from the added vitamin. The practice of correcting the vitamin values for the loss, as determined by recovery tests, seems to be justified

4 The method has been applied to the assay of the vitamin B<sub>1</sub> content of 19 foods and found to yield values which corresponded well with the results of biological tests

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## INTESTINAL CHANGES IN MONKEYS FED ON POOR RICE DIETS

BY

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[Received for publication, December 31, 1941 ]

As the result of experiments carried out in Coonoor during the years 1918-21, McCarrison (1921) reached the conclusion that 'the health of the gastro-intestinal tract is dependent on an adequate provision of accessory food factors' and he noted that 'experiments with animals have led us to expect that acute intestinal disorders will be among the commonest of the consequences of deficient and ill-balanced food' McCarrison's experiments, which were carried out on monkeys, pigeons and guinea-pigs, are fully described in his book 'Studies in deficiency disease' In the monkey experiments he used autoclaved diets which were grossly deficient in vitamins and other essential food factors, deficiency states were rapidly induced and were for the most part of an acute and fulminating nature Of 25 monkeys fed on the deficient dietaries 21 developed diarrhoea or dysentery and most of these died within a few weeks No animal survived for more than 2 to 3 months

While McCarrison's conclusions with regard to the vulnerability of the intestine in monkeys to defective feeding were fully justified on the basis of his experimental data, the diets used were more ill-balanced and deficient than any type of diet likely to be consumed by human beings Further, deficiency states as met with in man are usually of a more sub-acute or chronic type than those which he observed in his experimental animals It is thus difficult to draw conclusions as to the parallelism between the intestinal conditions observed in his monkeys and intestinal conditions occurring in human beings

The object of the present investigation was to observe the effect on monkeys of long-continued feeding on poor rice diets resembling those consumed by human

## SUMMARY

1 A simple method for the estimation of vitamin B<sub>1</sub> in foods, using the thiochrome reaction, has been described

2 Materials interfering in the thiochrome test were eliminated to a large extent by treatment with lead acetate and preliminary washing with isobutyl alcohol

3 Evidence has been produced to show that the incomplete recovery of added vitamin is due to (a) the loss occurring during the process of oxidation and (b) the inhibiting effect of unknown isobutyl alcohol 'test' solutions on the fluorescence due to the thiochrome formed from the added vitamin. The practice of correcting the vitamin values for the loss, as determined by recovery tests, seems to be justified

4 The method has been applied to the assay of the vitamin B<sub>1</sub> content of 19 foods and found to yield values which corresponded well with the results of biological tests

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TABLE I—*concl'd*

Diet A.	Per monkey per day	Diet A	Per monkey per day
Root vegetables	40 g	*Fruit	20 g
Other vegetables (raw)	40 „		

\* Given three days in the week, i.e. on Mondays Wednesdays and Fridays, about 50 g at a time

*Pulses*—Bengal gram (*Cicer arietinum*), black gram (*Phaseolus mungo*), green gram (*Phaseolus radiatus*) and dhal arhar (*Cajanus indicus*)—equal parts ground into flour

*Root vegetables*—Potato, carrot, sweet potato or turnips

*Other vegetables*—

Sunday	Bitter gourd ( <i>Momordica charantia</i> )
Monday	Cabbage
Tuesday	Cucumber or calabash cucumber
Wednesday	Lady's finger ( <i>Hibiscus esculentus</i> )
Thursday	Brinjal ( <i>Solanum melogena</i> )
Friday	Pumpkin
Saturday	Green plantain

*Fruit*—Plantain, mango, orange, apple or papaya

The atta, pulse, whole milk and butter or ghee were mixed and made into a 'chapatti' (unleavened cake). Half the 'chapatti' was given at 9 a.m. and the other half at 3 p.m. The other foods were fed separately in the raw state. Root vegetables were given in the morning and the other vegetables (raw) and fruit in the evening.

TABLE II

*Diet B*—Based on milled rice and containing supplementary foods in small quantities

Diet B	Per monkey per day	Diet B	Per monkey per day
Parboiled or raw milled rice	100 g	Coco nut oil	0.25 c.c.
*Vegetable	10 „	*Salt	0.5 g
*Pulse	7 „	Chillies (powder)	0.25 „
Gingelly oil	0.5 c.c.	*Tamarind	0.25 „

\* These were made into a soup by boiling and mixed with cooked rice. gingelly oil and coco nut oil were added. Half the above quantities was given at 9 a.m. and the other half at 3 p.m. The amount of food supplied was always somewhat in excess of requirements.



The monkeys on diet A consumed all the fruits and vegetables and practically all the milk. A variable proportion, usually about one-third, of the whole-wheat chapatti was often left unconsumed. The cooked rice and soup of diet B were mixed well, made into a bolus, and offered *ad lib* to the animals.

Diet A was superior to diet B in respect of nearly all the important food factors. The latter was deficient in vitamins A and C, vitamins of the B group and calcium.

The animals were kept in separate cages under hygienic conditions. They were housed in airy rooms, the temperature of which was kept fairly constant. Artificial heating was supplied during the cold season. The animals were set free for exercise every day in an enclosed run which provided plenty of space and sunshine.

The investigation was commenced in April 1938 and completed in March 1941. Altogether 84 animals were observed, 23 on diet A and 61 on diet B.

#### OBSERVATIONS

No significant differences in the state of nutrition of the two groups were observed during the first few weeks. But as the experiment progressed, striking differences were noticed in the health and well-being of the two groups. Most of the animals in group A increased steadily in weight, while the weight of those on the rice diet remained stationary or declined. As regards weight, there was very considerable individual variation.

The condition of the animals in group A was in general satisfactory. They were active and ate their food well. Some animals in this group survived for over 2 years in good condition. In general they remained fairly free from disease. Some animals suffered from relatively mild attacks of diarrhoea from which they recovered easily. Dysentery, as shown by blood and mucus in the stools, also occurred in 8 members of the group. Usually they recovered rapidly and no death occurred from this cause. One animal in group A lost weight steadily and on post-mortem examination an adeno-carcinoma of the stomach was revealed.

The monkeys in group A, when out at exercise, were active and playful, climbing all over the enclosure. Those in group B, on the other hand, soon became listless and lost energy and much of the normal simian interest in their surroundings. They spent much of their time picking one another's pelts. On approaching the enclosure, it was usually possible to distinguish from a distance of about 25 yards which group was out at exercise, from the behaviour of the animals.

Some of the animals fed on the rice diet became progressively weak and died within 6 or 8 weeks, others survived for 6 to 12 months. In some animals the survival period was extended by the addition of supplements, described below, to the diet when the animal appeared to be in a bad state. In animals surviving for several months, a fairly constant clinical picture made its

appearance, though there was much individual variation as to the time of onset. The appetite was impaired and attacks of diarrhoea occurred. The stools were small, pale in colour, and varied in number from 4 to 10 per day, microscopic examination of the stools revealed undigested food particles degenerating epithelial cells and a large number of bacteria. Mucus and blood were not present. In untreated cases, the animals usually became steadily worse and died. As the aim of the experiment was to study the effects of chronic diet deficiency, supplements of milk (100 c c), sprouted gram (100 g) and one ripe plantain a day were given to some monkeys with diarrhoea in addition to the basal diet, for a few weeks at a time, the object being to extend the period of survival. In animals which had not been fed for prolonged periods on the deficient diet and were in a fairly good general condition, the provision of these supplements usually checked the diarrhoea. The supplements were withdrawn as soon as the condition of the animal improved. As the period on the deficient diet increased the attacks of diarrhoea tended to become more frequent and prolonged, until finally a condition of chronic diarrhoea, leading to emaciation and death, supervened in many animals in the group. In the later stages of the disease, the supplements were without effect in checking the diarrhoea and improving the general condition. The incidence of attacks of diarrhoea in group B was about three times as great as in group A. The average duration in days of each attack of diarrhoea was considerably greater in the former.

During certain seasons, dysentery, evidenced by blood and mucus in the stools, occurred in both groups, presumably due to an infection carried by flies. While animals in group A usually got over the attack quickly in group B it showed a tendency to pass into the sub-acute or chronic stage.

In addition to the gastro-intestinal disorders some animals in group B showed signs indicative of deficiency disease. These included catarrhal ophthalmia, xerosis of the conjunctiva, blepharitis lachrymation, swelling of the eye-lids (see Plate IX, fig 20), pyorrhoea alveolaris, spongy and bleeding gums, ulceration of the gums wrist-drop (see Plate IX, fig 18), spastic or flaccid paralysis of the limbs and chronic ulcers on the hind limbs. Two conditions require special mention. These are disorders of the skin and oedema. The hair became coarse, sparse and 'staring'. Some chronically deficient animals showed a symmetrical denudation of the hair over both hands and feet. The skin in these areas was rough and scaly, and showed hyperpigmentation and branny desquamation (see Plate VIII, figs 13 to 16). The condition was in some degree suggestive of the cutaneous manifestations of pellagra. The tail also showed similar changes, in addition chronic indolent ulcers were frequently present especially over the feet.

A few animals showed oedema, which was peculiar in its onset and distribution. The oedema tended to appear suddenly, usually over the lower part of the face. It also involved the scrotum (see Plate IX, fig 17). Some monkeys showed swelling of the hands and, occasionally of the feet. The addition of supplements usually cleared up the oedema. The neurological manifestations and oedema were more marked in animals (15 in number) fed on raw milled rice which has a much lower vitamin B<sub>1</sub> content than parboiled milled rice (Aykroyd, Krishnan,

Passmore and Sundararajan, 1940) These animals usually went down-hill rapidly, and none survived for more than 23 weeks

The terminal clinical picture, shown by a large proportion of the group, was one of great emaciation, marked prostration, and chronic diarrhoea. In this condition, the animals lost appetite for food and assumed a peculiar hunched posture, bending the upper part of the body and putting the head in between the hind limbs with the hands behind the neck.

The various lesions of the skin, eyes and gums described above, and cedema, did not occur in group A.

#### AUTOPSY FINDINGS

A complete post-mortem examination was carried out on 15 animals in group A and 40 in group B. Most of those from group A examined were killed in a healthy state. In group B, the animals were usually killed when moribund. Pieces of tissues were taken for histopathological study as soon after death as possible to avoid post-mortem changes.

The following description applies in general to the chronically deficient animals in group B —

The animal was much emaciated and the face had a pinched appearance. The skin and hair showed the appearances described above. One animal showed aphthous ulceration of the gums (see Plate IX, fig. 19) similar in appearance to that ascribed to 'vitamin M' deficiency by Langston, Darby, Shukers and Day (1938).

In the abdomen, there was very little subcutaneous, retroperitoneal or omental fat. Distension of the stomach and intestines was occasionally noticed. The omentum and mesentery were thin. The mesenteric and ileo-cæcal glands were enlarged. Intussusception of the descending type was occasionally noticed, but this occurred in both groups.

On opening the thorax, the lungs were found collapsed and, in some cases, adhesions between the parietal and visceral pleura were present. The lungs, four-lobed on the right side and three-lobed on the left, showed varying degrees of pigmentation. The heart was smaller in size than normal. In one instance, a caseating tubercular gland was noticed at the hilum of the right lung. Evidences of patchy consolidation (non-tubercular) were present in a few cases. It is of interest to note that tuberculosis was a rare complication in these experiments, because monkeys used for experiment in Europe and America frequently fall victim to this disease. The relative absence of tubercular infection was probably due to the facts that the animals were used for experiment immediately after being caught in the jungle, and located in a healthy rural area, 6,000 feet above sea-level.

The internal appearances of the gastro-intestinal tract were interesting. The tongue was furred and the papillæ were prominent, occasionally, however, it showed a glazed appearance. The mucous membrane of the pharynx and

PLATE V.

[All the photomicrographs were taken with 'Miflex' (Zeiss)]

Figs 1 to 4—Photomicrographs of the lower part of small intestine of monkeys fed on rice diets (group B), showing varying degrees of atrophy



FIG 1



FIG 2

Figs. 1 and 2 show the 'withering of the villi' and flattening of the villi respectively  $\times 50$

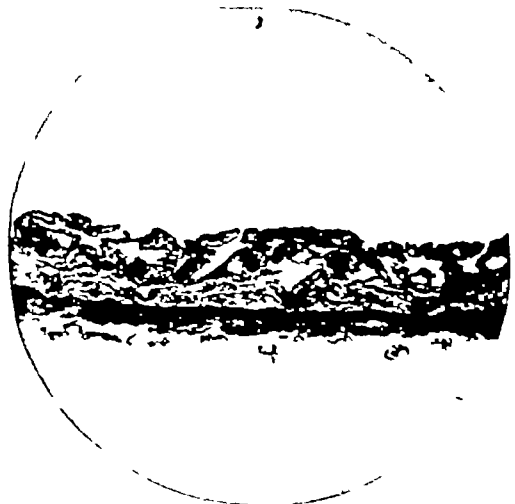


FIG 3



FIG 4

Figs. 3 and 4 show degenerative changes in the glands of Lieberkühn, which are greatly reduced in number

## PLATE VI

Figs 5 and 6—Photomicrographs of the lower part of small intestine of monkeys fed on the diets (group B), showing varying degrees of atrophy

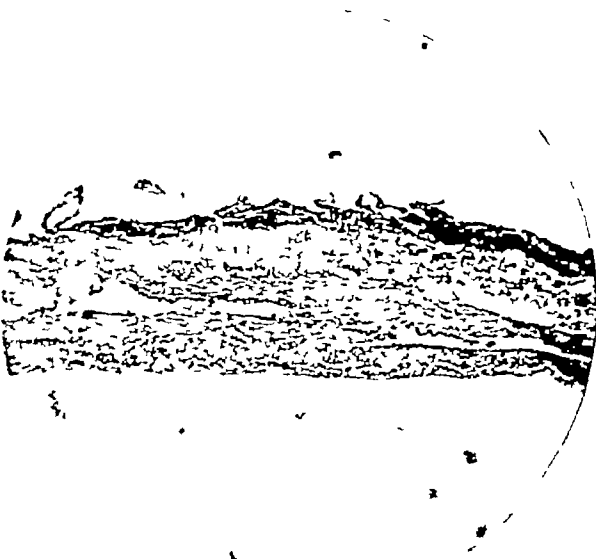


FIG 5



FIG 6

Figs 5 and 6—Note the atrophic mucous membrane, which is only a few cells deep in Fig 5, and the atrophic lymphoid follicle in Fig 6  $\times 50$



FIG 7



FIG 8

Figs 7 and 8—Photomicrographs of upper (Fig 7) and lower (Fig 8) parts of small intestine of a monkey fed on diet A showing normal appearances  $\times 50$

PLATE VII.



FIG 9



FIG 10

Figs 9 and 10 —Photomicrographs of upper part of small intestine of monkeys in group B showing 'withering of the tips of the villi' (Fig 9) and flattening of the villi and atrophy of the mucosa (Fig 10)  $\times 50$



FIG 11



FIG 12

Figs 11 and 12 —Large intestine of monkeys in group B showing acute (Fig 11) and sub-acute (Fig 12) dysenteric ulceration  $\times 50$



FIG 13



FIG 14

Figs 13 and 14 —Photographs of the hands and feet (Fig 13) and tail (Fig 14) of a monkey in group A showing normal appearances



FIG 15

Figs 15 and 16 —Photographs of hands as they appear after 100 days of treatment with 1% solution of 2,4-dinitrophenol



FIG 16

Fig 16 shows the tail of a monkey in group A showing normal appearances



FIG 17



FIG 18



FIG 19



FIG 20

FIGS. 17 to 20 —Photographs of monkeys in group B showing edema of the face and scrotum (Fig 17), wrist-drop (Fig 18) ulceration of the gums (Fig 19) and edema of the lower eye lids (Fig 20) The animal shown in Fig 20 developed marked flaccid paralysis of all limbs





œsophagus were pale. The tonsils were atrophic. The mucous membrane of the stomach was also pale and usually thinner than normal, in some cases, it showed superficial erosions and petechiæ. The duodenum was catarrhal and the mucosa was lined by a thick layer of mucus. Chronic gastric or duodenal ulceration was not present in any case. The jejunum and ileum showed varying degrees of atrophy, this being more marked in the lower part of the ileum, from about 10 to 12 inches above the ileo-cæcal valve downwards. The mucous membrane of the gut was soft, œdematous and lined by a layer of thick mucus. On washing the gut, it was observed that the transverse folds of the mucous membrane were not prominent in the jejunum and were entirely lost in the lower ileum. In the latter, the atrophic Peyer's patches were rendered very prominent by the atrophic changes in the mucosa. The muscular layers also showed varying degrees of atrophy and, in a well-marked case, the ileum looked papyraceous. Instead of showing the normal pinkish appearance of the gut, as seen in transmitted light, the ileum was palish-white in colour. Petechial hæmorrhages were present in the small intestine in a few animals.

The mucous membrane of the large intestine did not show similar atrophic changes. Evidences of acute, sub-acute, or chronic bacillary dysentery were present in the large gut in animals which had suffered from the disease. The solitary lymph follicles were prominent in some cases.

The liver was atrophic and in some cases showed a 'nutmeg' appearance. There were milary tubercles in one animal. In another, the suprarenals were enlarged to nearly thrice the normal size and, on section, showed hæmorrhages in the medulla. The kidneys were pale and in some cases showed retention cysts. Other internal organs showed no noteworthy macroscopic changes.

Gross gastro-intestinal lesions, of the type described above, were not met with in group A. The mucosa usually showed some catarrh and was covered by mucus. In one instance, the stomach showed superficial erosions of the mucosa and petechial hæmorrhages. The large intestines showed evidences of bacillary dysentery in those animals which had suffered from attacks of the disease.

#### HISTOPATHOLOGY OF THE GASTRO-INTESTINAL TRACT

(1) *Group B*—The tongue showed no evidences of gross atrophy of the epithelium. In some instances, however, the epithelium was slightly thinner than normal. In few cases, there was hyperkeratinization of the lining epithelium of the tongue and œsophagus.

The mucosa of the stomach showed lymphocytic infiltration of the superficial parts of the mucosa, atrophy of the gastric glands and superficial erosions in some cases. In a few, the mucosa and sub-mucosa showed petechial hæmorrhages. Congestive and hæmorrhagic changes were occasionally noted in the duodenum.

Serial sections of the intestines of animals surviving for varying periods on the deficient diet were examined. In animals fed on the rice diets for about 10 weeks, the epithelium covering the villi showed degeneration. The lymphoid

elements were reduced in number. In cases surviving for longer periods, the villi were almost acellular, with the covering epithelium lost. These appearances are similar to the 'withering of the villi' described by Mackie and Fairley (1929) in sprue. Specimens removed from animals suffering from prolonged diarrhoea showed flattening of the villi and decrease of the reticular tissue. In these cases, the mucous membrane was only a few cells deep. Degenerative changes in the glands of Lieberkuhn appeared only in the most protracted cases of diarrhoea. The protoplasm of the affected glands was granular, and their nuclei were swollen. The glands did not take the stain properly and disappeared completely in places, thus reducing considerably the number of glands in the mucous membrane. The atrophy of the glandular elements varied greatly in different animals, and varying degrees of the condition were noticed in the same section. The muscularis mucosa was less prominent than normal. Oedema and congestion of the mucous membrane were also observed in some of the cases of relatively short duration. It is interesting to note that at no stage were inflammatory changes observed in the mucosa or sub-mucosa.

The sub-mucous coat showed dilated and congested vessels and small collections of effused red blood cells. These petechial hæmorrhages were seen even in cases which showed macroscopically no evidence of hæmorrhagic diathesis.

The essential change in the muscular coat of the small intestine was atrophy. But here, in contradistinction to the mucosa, the change was essentially a reduction in the volume of the muscular tissue, the individual fibres and their nuclei which remained appearing normal. The serous coat showed no abnormality beyond congestion in some instances.

Degenerative changes were constantly present in Auerbach's plexus. In most cases the plexus was enlarged and oedematous, and hence easily recognized in the sections. Varying degrees of degenerative changes, from cloudy swelling to complete degeneration, were found in the ganglion cells. The number of ganglion cells involved in the degenerative process and their distribution in the plexus showed great variation in the several specimens. In chronic cases, normal ganglion cells were rarely seen and the plexus was represented by empty spaces containing skeletons of dead or degenerating ganglion cells, round cells, fibroblasts and glial cells.

Changes characteristic of sub-acute or chronic bacillary dysentery were observed in the large intestine in some cases. Only in one case were degenerating amoebæ found in the sub-mucosa. The large intestine showed no evidence of the marked atrophic changes noticed in the small intestine. The mucous glands showed slight atrophy in some cases. Auerbach's plexus, however, showed changes similar to those described in the small intestine.

(2) *Group A* —The atrophic changes in the small intestine described above were not evident in group A. Ulceration of the mucosa of the large gut was, however, present in some animals in this group. Study of the serial sections suggested that this condition was not so advanced or chronic as in group B.

Plates V to VII illustrate the changes described in the preceding sections.

## HISTOPATHOLOGICAL CHANGES IN OTHER VISCERA AND TISSUES

The following changes were observed, singly or in combination with others, in various animals in group B —

*Liver*—Fatty degeneration, necrosis of the parenchymal cells around the central vein, icteric necrosis of the parenchymal cells, pericholangitis, miliary tubercles

*Lungs*—Broncho-pneumonia and miliary tuberculosis of the lungs, caesating hilum gland

*Endocrines*—Cystic thyroid, slight increase of perivascular connective tissue in the parathyroid, hæmorrhages in the cortex and medulla of the suprarenals

*Skin and epithelium*—Slight hyperkeratinization of the epidermis and mouths of the hair follicles, hyperpigmentation (more marked over hands and feet), slight hyperkeratinization of the epithelium lining the vagina

*Nervous system*—Slight degeneration of the posterior columns and posterior nerve-roots in the spinal cord, occasionally, similar changes in the lateral columns, slight myelin degeneration of the peripheral nervous system.

In group A, one animal showed broncho-pneumonia of the lungs and another had emphysema. Secondary malignant deposits were noticed in the liver of the animal, referred to above, which showed a malignant growth in the stomach

## HÆMATOLOGICAL FINDINGS

Complete hæmatological investigations were carried out on all animals in both groups. Some animals in group B showed slight secondary anæmia, a few developed marked hypochromic, microcytic anæmia. Macrocytic hyperchromic anæmia was not observed in a single instance in animals fed on the deficient diets for prolonged periods. Smears taken from the bone-marrow showed a normoblastic reaction in cases with secondary anæmia. A megaloblastic reaction of the bone-marrow was not observed in any case. Fractional test meals, however, showed a high incidence of hypochlorhydria in the stomach.

## DISCUSSION

Two types of change were thus noticed in the gastro-intestinal tract of monkeys fed on the rice diets. The changes in the small intestine were degenerative in nature and non-inflammatory. On the other hand, the lesions of the large intestine were inflammatory in type. Both groups of animals were kept under the same environmental conditions, the only difference being the diet given. It seems clear that the atrophic changes found in the small intestine in group B were due solely to the deficient diet since similar changes were not evident in animals

in group A fed on the well-balanced diet. In starvation generalized atrophy of all the internal organs occurs, but not atrophy limited to the lower part of the small intestine.

The lesions of the large intestine, which were observed in animals in both groups, were evidently the result of a superimposed infection. Bacillary dysentery is not uncommon in Coonoor, particularly in the hot season, and the monkey houses and food attracted numerous flies at this time of the year. Histologically the lesions were typical of bacillary dysentery. In one case in which a cultural examination of the stools was carried out, a pure culture of *B. dysenteriae* (Flexner) was isolated.

While the intestinal condition dominated the clinical picture, group B in general showed multiple signs of deficiency disease. This is to be expected, since the diet was deficient in many essential food factors. The presence of multiple hæmorrhages in the different organs observed microscopically suggested that the animals were in a state of subclinical scurvy. On the basis of the observations recorded, however, the intestinal changes cannot be ascribed to deficiency of any particular vitamin or other food constituent. Further work will be necessary to establish any such association.

While the above experiment was in progress, an interesting paper was published in the *East African Medical Journal* by McKenzie (1940). He described a clinical condition occurring in African labourers subsisting on very poor diets, which appears to be almost identical with that observed in the monkeys. The name 'nutritional diarrhoea' was given to this condition. The gastro-intestinal symptoms and the microscopic appearances of the gut in these cases were closely similar to those described above. One noteworthy observation was that the incidence of 'nutritional diarrhoea' among estate labourers fell remarkably, with an improvement in the diet and living conditions on the estates.

The high incidence of gastro-intestinal disorders, particularly diarrhoea and dysentery, in India, and the increased death rate due to these causes in times of famine are well known. In a report on the recent famine in Hissar, Punjab, Nicol (1941) points out that there was a striking increase in the incidence of 'dysentery and diarrhoea' during the period of famine. This is additional evidence to show that the digestive tract is affected by malnutrition. It is to be observed that the death rate from 'dysentery and diarrhoea' in the Punjab, as recorded in the Annual Reports of the Public Health Commissioner with the Government of India, is usually from one-fourth to one-sixth that of Orissa, the Central Provinces and Madras. The Punjab is a wheat-eating province, while in the latter provinces rice is the principal or an important staple.

Inquiries among clinicians suggest that a clinical picture similar to that shown by the ill-fed monkeys and reported by McKenzie in East Africa is often seen in malnourished patients in India. Cases of chronic diarrhoea of obscure ætiology which do not respond to any kind of treatment and slowly waste away and die are seen in the 'chronic wards' of any teaching hospital in South India. No systematic investigation of this condition has as yet been carried out. It would

be interesting to know whether such cases exhibit atrophic changes in the gut, similar to those described above

Manson-Bahr (1941), in a recent article reviewing the ætiology of the sprue syndrome, points out that 'sprue' is not a separate clinical condition, but an assembly of clinical phenomena due to damage to the mucosa of the small intestine. The syndrome may be produced by a number of diverse surgical and medical conditions, e.g. gastro-jejuno-colic fistula (de Rivas, 1930, quoted by Manson-Bahr, 1941, Fairley and Kilner, 1931), ulceration of the ileum, blockage of the lymphatic supply of the bowel, as in tabes mesenterica, lymphadenoma lympho-sarcoma of the mesenteric glands (Fairley and Mackie, 1937), malignant disease of the small gut (Manson-Bahr, *loc cit*). The term 'chronic jejuno-ileal inefficiency' has been introduced to denote this condition. A further possible cause of this condition is a diet deficient in essential food factors, such as the 'poor rice diet' supplied to the monkeys in these experiments. The syndrome presented by the monkeys, however, differed from that of typical tropical sprue, in that the typical lesions of the tongue and macrocytic anæmia were absent.

Wills and her co-workers (Wills and Bihmoria, 1932, Wills and Stewart, 1935) have reported the development of a nutritional macrocytic-hyperchromic anæmia in monkeys fed on diets resembling those consumed by patients suffering from 'tropical macrocytic anæmia' in Bombay. The rice diet given to monkeys in this investigation resembled that used by the above workers, but none of the animals showed any evidence of macrocytic anæmia. The anæmia which resulted was, as already pointed out, microcytic and hypochromic.

#### SUMMARY

Monkeys fed on diets based largely on milled rice and containing supplementary foods in small quantities, similar in composition to those consumed by the poor rice-eaters in India, developed a chronic diarrhoea accompanied by atrophic changes in the small intestine, especially of the lower third. Histological examination showed varying degrees of degenerative changes in the different layers of the gut and in the intramural nerve plexus. These changes did not occur in animals fed on a good diet based on whole wheat, milk, and vegetables.

In both groups, inflammatory lesions resulting from a superimposed bacillary dysenteric infection were sometimes present in the large intestine.

#### ACKNOWLEDGMENT

The author wishes to acknowledge the keen interest shown in these experiments by Captain R. Passmore, I.M.S., Assistant Director, Nutrition Research Laboratories, Coonoor.

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## THE GROWTH-PROMOTING VALUE OF EGGS

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[Received for publication, November 17, 1941 ]

EGGS are generally recognized as an excellent supplement to the human diet, since they possess most of the essential elements which are either absent or deficient in the cereals that normally provide the major bulk of man's food. In countries where the standard of living is high, eggs are widely consumed, even though other valuable supplementary foods, such as milk, meat and fish, form a considerable portion of the diet. In India, however, where the purchasing power of the individual is, on the average, extremely low, the diet is usually very deficient in well-balanced proteins and other essential food factors. The poor quality of this diet is due to an excessively high consumption of cereals and a low consumption of foodstuffs of animal origin.

Though eggs are generally accepted as being equivalent in nutritive value to milk, it is surprising to note the disparity in the amount of data available in regard to the values of these products. There is a large volume of literature concerning the value of milk both for human beings and animals but relatively little authentic information concerning the value of eggs. Experimenting upon animals, Mitchell and Carman (1924, 1926) found that the protein of cooked eggs was superior to that of all other foodstuffs, even including milk. Sumner (1938), Sumner, Pierce and Murlin (1938) and Sumner and Murlin (1938) confirmed the superior value of egg proteins by experiments on humans, though in the adult human, the superiority was less marked than in young or adult rats. By taking one egg a day, as a basis of comparison, Kon (1940) assessed the value of eggs in human nutrition and compared their nutritive value with that of milk and other protective foods. Comparing the dietary value of an egg with that of half a pint of milk, he found little difference in the calories, protein, fat, vitamin A



vitamin B and riboflavin content While it is true that milk is a better source of calcium than eggs, the latter are much richer in vitamin D and in iron, both of which are deficient in milk

In nutritional experiments with young rats fed on various typical Indian diets, McCarrison (1927) showed that the nutritive value of these diets ranged themselves in the following descending order Sikh, Pathan, Mahratta, Kanarese, Bengali and Madrassi More recently, Aykroyd and Krishnan (1937) found that the average weekly increase in weight of young rats over a period of 10 weeks on a typical Madrassi diet was 2.8 g, whereas the same diet supplemented with the equivalent of an egg a day (1.5 oz albumin and yolk) gave a weekly increase of 6.8 g Whole-milk powder and skimmed-milk powder, when added at the rate of 1.5 oz daily, gave average weekly increases of 6.7 g and 7.5 g respectively

The following investigations have been carried out to determine —

- (1) The number of eggs that should be added to a Bengali village diet to produce an optimum rate of growth in rats
- (2) The value of soya beans as a partial replacement of egg supplements in a Bengali village diet

The Bengali diet selected was that of the small agriculturists in the district of Dinajpur, Bengal, particulars of which have been taken from a diet survey carried out by Mitra (1939) This diet contains only small quantities of pulses, vegetables, and fish, together with an unduly high proportion of rice As the protein content of rice is poor both with regard to quality and quantity, the ration is very badly balanced, its nutritive ratio being thirteen, whereas well-balanced diets should have a nutritive ratio of seven The composition of this Bengali diet is given in Table I The quantities given correspond to the daily intake in ounces per adult man

TABLE I

*Bengali village diet (daily intake in ounces)*

Rice	Pulses	Leafy vegetables	Potatoes	Brinjals	Tara	Mustard oil	Common salt	Fish
25.0	0.4	0.2	3.5	1.75	1.75	0.3	0.1	0.7

#### EXPERIMENTAL

In all the tests, 2 male and 2 female four-week old rats were fed on the experimental diets for a period of 10 weeks The sexes were caged separately and the average weights of the males and females in each group were comparable at the commencement of each experiment Sufficient food for 3 days for each of the groups was prepared at each mixing All the food mixtures were cooked for half an hour on the water-bath before feeding in order that they should resemble the human dietary as closely as possible The rats were fed each afternoon, the

amount fed being slightly in excess of appetite. The residue was collected each morning and at the end of each week the actual amount of food consumed was calculated by subtraction of the total dry weight of the residue from the total weight fed. Distilled water was given to drink and also used for making up the food. In each experiment one group of rats (2 males and 2 females) was fed on the stock diet used in rat experimental work at the Animal Nutrition Research Section of this Institute. This diet consists of 97 parts whole-wheat flour, 1 part dried brewers' yeast, 1 part common salt and 1 part ground limestone, supplemented with separated milk (140 c.c. per 100 grammes of stock mash) and green grass. This stock diet, with its protein content of 13.7 to 14.0 per cent on a dry matter basis, had been proved very satisfactory under test. The rats were weighed individually every week.

In the first series of experiments, carried out in duplicate the following diets were tested out —

Diet number	Designation	Composition
1	Stock	Stock mash <i>plus</i> milk and green food
2	B	100 parts Bengali diet
3	B + Ca	99.5 parts Bengali diet <i>plus</i> 0.5 parts ground egg shell.
4	100B + 12E	100 parts Bengali diet <i>plus</i> 12 parts egg
5	100B + 12E + Ca	100 parts Bengali diet <i>plus</i> 12 parts egg <i>plus</i> 0.5 per cent ground egg shell
6	100B + 25E	100 parts Bengali diet <i>plus</i> 25 parts egg
7	100B + 25E + Ca	100 parts Bengali diet <i>plus</i> 25 parts egg <i>plus</i> 0.5 per cent ground egg shell
8	100B + 50E	100 parts Bengali diet <i>plus</i> 50 parts egg
9	100B + 50E + Ca	100 parts Bengali diet <i>plus</i> 50 parts egg <i>plus</i> 0.5 per cent ground egg shell.

The chemical compositions of diets Nos 2, 4, 6 and 8 are given in Table II. It will be seen from these figures that the Ca/P ratios of the Bengali diet and of the egg-supplemented Bengali diets are very wide. Experimenting upon rats, Simmonds (1924) recommended a Ca/P ratio of 1.063 and Bethke and Edgington (1927) and Bethke (1933) found the best results were obtained with a range between 1.055 and 1.1. However, experimental workers generally agree that the possible range of variation depends to a large extent on the vitamin D supply and that, with an abundant supply of vitamin D, rats can thrive on rations with wider Ca/P ratios than those quoted. In the experiments under report, the rats were protected against vitamin D deficiency by exposing them to the sun for 40 minutes every alternate morning. As the calcium contents of the different diets

were much lower than the recommended figures, supplements of 0.5 per cent ground egg-shell were added to diets Nos 3, 5, 7 and 9. The addition of 0.5 per cent of the ground egg-shell raised the calcium contents of the diets by 0.19 per cent. The calcium-supplemented diets had a Ca : P ratio of 1 : 0.8.

TABLE II

*Percentage composition of experimental diets*

Diet	Moisture	Crude protein	Ether extract	Ash	Nitrogen free extract	Nutritive ratio	Calories per 100 g	Calcium	Phosphorus	Ca : P	Crude protein on dry basis
B	20.0	6.0	1.0	1.30	71.7	12.3	328	0.036	0.150	1 : 4.2	7.5
100B + 12E	25.8	6.9	2.0	1.27	64.0	9.9	309	0.038	0.163	1 : 4.3	9.3
100B + 25E	30.8	7.5	2.9	1.24	57.6	8.6	294	0.042	0.174	1 : 4.1	10.8
100B + 50E	38.0	8.5	4.2	1.20	48.1	6.8	271	0.044	0.190	1 : 4.3	13.7

As experiment 2 was a repetition of experiment 1 and as the results obtained in each experiment were very similar for each of the diets under test, the combined average results of the two experiments are given in Table III. The combined growth results of the males and females on each diet are given in Graphs 1 and 2 respectively.

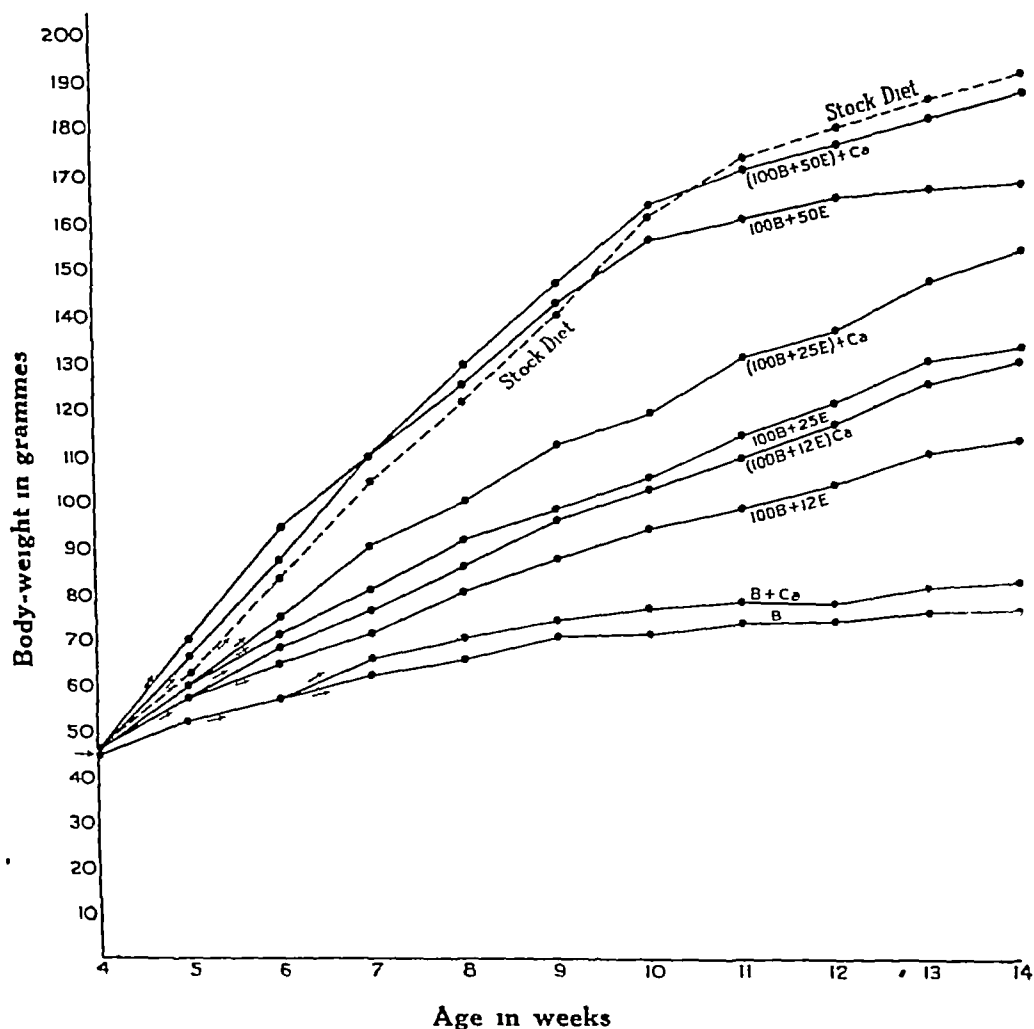
TABLE III

*Protein consumption and growth rate*

Diet	Percentage of protein on dry basis	AVERAGE WEEKLY INCREASE IN WEIGHT (g) OF THE YOUNG RATS DURING 10 WEEKS' EXPERIMENTAL PERIOD	
		Male	Female
B	7.5	3.3	2.0
B + Ca	7.5	4.0	2.5
100B + 12E	9.3	7.1	4.8
100B + 12E + Ca	9.3	8.8	6.3
100B + 25E	10.8	9.0	6.4
100B + 25E + Ca	10.8	11.1	7.6
100B + 50E	13.7	12.6	8.3
100B + 50E + Ca	13.7	14.0	9.8
Stock diet	13.8	14.9	10.0

The average weekly gains from 4 to 14 weeks of 3.3 g and 2.0 g for the male and female rats fed on the Bengali diet were much below normal. Graphs 1 and 2

GRAPH 1  
Combined growth curves of male rats  
(Experiments 1 and 2)



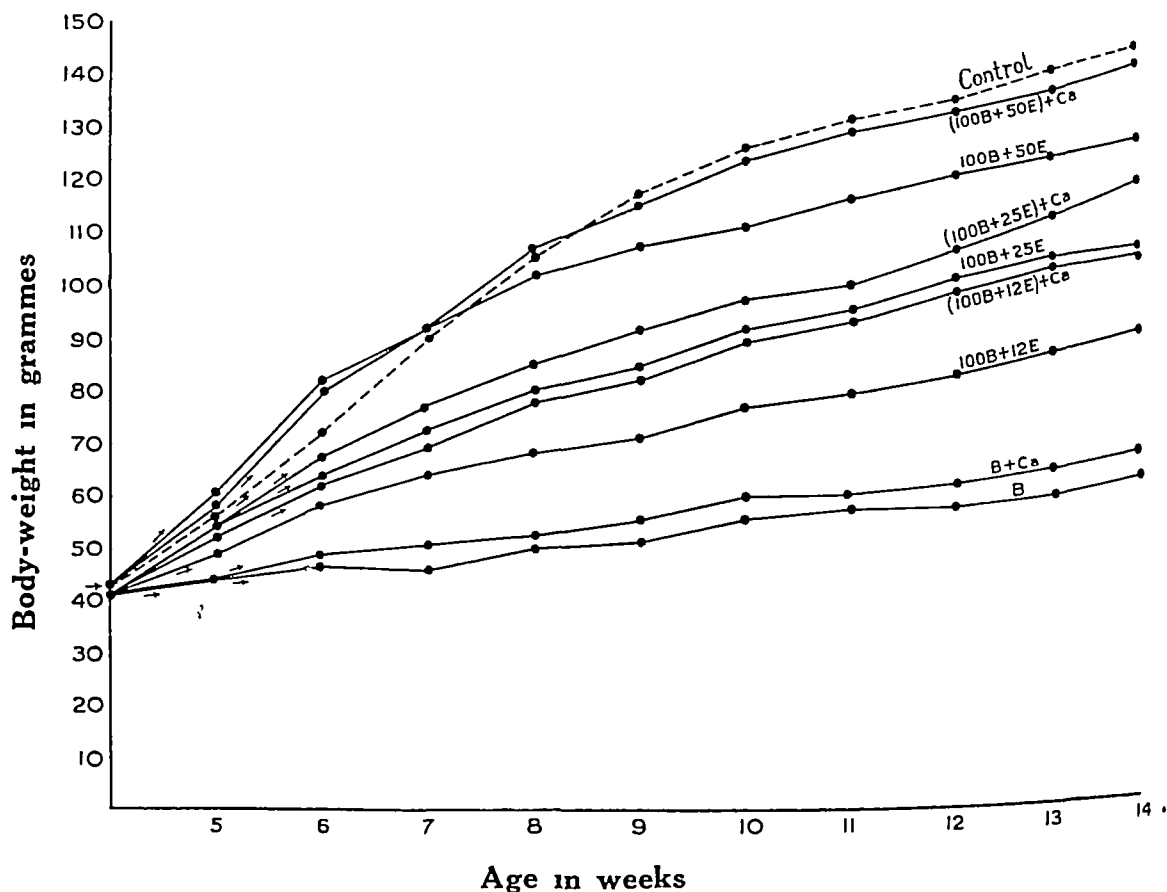
show that from 9 to 14 weeks the growth curves remained practically horizontal for both sexes fed on the Bengali diet. The B + Ca diet was only slightly

superior to that of the B diet. The addition of 12 parts egg to the Bengal diet resulted in a marked improvement in the growth curve, for the average weekly gains made by the males and females were 7.1 g and 4.8 g respectively. The addition of 25 parts egg raised the average weekly gains to 9.0 g and 6.4 g respectively. The average weekly increases in weight of the male and female rats on the 100B + 50E diet were 12.6 g and 8.3 g respectively. The

GRAPH 2

*Combined growth curves of female rats*

*(Experiments 1 and 2)*



supplements of ground egg-shell, though of little value in the case of the Bengal diet, had a very beneficial influence on the egg-supplemented diets. In the case of 100B + 12E + Ca diet, the average weekly increases were almost as great as in the 100B + 25E diet. The weekly weights of the rats on the 100B + 50E + Ca diet were practically the same at each weekly weighing as those of the rats on the control diet.

The rats in experiment 1 were maintained in the laboratory on the same diets for another 6 weeks after the completion of the experiment proper. During this period, the rats on all the diets except the Bengali diet remained healthy but those on the latter diet all began to show an abnormal eye condition from 12 weeks onwards. The eyes first became inflamed, later the corneæ became opaque and eventually the animals were blind. Two rats each from the control group, the B group and 100B + 50E group were killed at 16 weeks and their livers quantitatively tested for vitamin A. No vitamin A was found in the livers of the rats fed on the Bengali diet, whereas the rats fed on the other two diets had each an average of 25 blue units of vitamin A per g of liver. Four out of the total of 16 rats fed on the Bengali diet and the Bengali diet *plus* egg-shell had abnormal swellings in the sub-maxillary gland, whereas no such condition was noticed in any of the rats from the other groups.

Table IV has been calculated on the basis of Aykroyd's (1938) Indian standards for children from 6 to 9 years. The table gives the amounts of the different diets that would have to be consumed daily to give a daily protein intake of 60 g. Based on these figures, the daily food consumption of the Bengali diet is excessively high, for 1,000 g food supply yields an energy value of 2,972 calories, whereas the recommended figures for a child of 6 to 9 years are only 1,300 to 1,600 calories. The caloric value for the 2 diets, 100B + 12E and 100B + 25E, are also considerably higher than normal requirements, while the 100B + 50E diet is in fairly close agreement with Aykroyd's figures. The table also gives the number of eggs, each with internal contents (white + yolk) weighing 45 g that would have to be fed along with the various egg diets to give a daily consumption of 60 g protein.

TABLE IV

*Calorific values and number of eggs consumed to give  
a daily protein intake of 60 g*

Name of diet	Consumption in g	Calories	Number of eggs required
B	1,000	2,972	
100B + 12E	869	2,444	2 1
100B + 25E	800	2,132	3 5
100B + 50E	706	1,747	5 2

Though the results with the 100B + 50E diet can be considered very satisfactory as regards growth rates, it is necessary to remember that eggs are relatively expensive and that the ordinary villager is too poor to purchase 5 eggs daily for each member of his household. Even were he to keep fowls to increase his purchasing power, he would undoubtedly prefer to sell his produce in order to increase his cash income. Also, though the addition of ground egg-shell to the

various egg-supplemented diets proved beneficial, it is unlikely that such a type of supplement would be readily adopted by human beings, even though the palatability of the food was not unduly altered

In the second series of experiments, it was decided to try out the substitution of various levels of soya beans for the egg supplements. Soya beans were selected in preference to other vegetable protein supplements, as it is generally accepted that their proteins have a higher biological value than those from other vegetable sources. In China, the soya bean is very widely used as a substitute for cow's milk and its use in other countries, both in the diets of the man and the animal, has been considerably extended in recent years. Moreover, soya beans are richer in calcium than most other pulses. Adolph and Cheu (1932), from calcium balance studies, found that cow's milk and soya bean curd were equally effective in supplying calcium in the Chinese diet.

Two identical experiments (experiments 3 and 4) were carried out to investigate the value of soya beans. In these, the standard stock diets were again used as controls and the protein levels of all the supplemented diets were kept constant. Except in the case of the control fed groups, parallel groups were fed each of the diets along with 0.5 per cent ground egg-shell. Details of the diets used are given below —

Diet number	Designation	Composition
10	B	100 parts Bengali diet
11	B + Ca	99.5 parts Bengali diet <i>plus</i> 0.5 parts egg shell
12	B + S	84.2 parts Bengali diet <i>plus</i> 15.8 parts soya
13	B + S + Ca	84.2 parts Bengali diet <i>plus</i> 15.8 parts soya <i>plus</i> 0.5 per cent egg shell
14	100B + 12E + S	88.2 parts (100 parts Bengali diet <i>plus</i> 12 parts egg) <i>plus</i> 11.8 parts soya
15	100B + 12E + S + Ca	88.2 parts (100 parts Bengali diet <i>plus</i> 12 parts egg) <i>plus</i> 11.8 parts soya <i>plus</i> 0.5 per cent egg shell
16	100B + 25E + S	92.7 parts (100 parts Bengali diet <i>plus</i> 25 parts egg) <i>plus</i> 7.3 parts soya
17	100B + 25E + S + Ca	92.7 parts (100 parts Bengali diet <i>plus</i> 25 parts egg) <i>plus</i> 7.3 parts soya <i>plus</i> 0.5 per cent egg shell
18	100B + 50E	100 parts Bengali diet <i>plus</i> 50 parts egg
19	100B + 50E + Ca	100 parts Bengali diet <i>plus</i> 50 parts egg <i>plus</i> 0.5 per cent egg shell
20	Stock	Stock mash <i>plus</i> milk and green food

Table V gives the composition of the new experimental diets Nos 12 14, and 16 —

TABLE V

*Percentage composition of diets*

Diet.	Moisture	Crude protein	Ether extract.	Ash	Nitrogen free extract	Nutritive ratio	Calories per 100 g	Calcium	Phosphorus	Ca P	Crude protein on dry basis.
B + S	18.5	11.2	3.5	1.85	64.9	6.5	348	0.09	0.24	1.264	13.7
100B + 12E + S	22.5	10.7	3.7	1.68	61.4	6.5	330	0.08	0.23	1.287	13.8
100B + 25E + S	28.9	9.8	3.9	1.50	55.9	6.6	306	0.07	0.21	1.300	13.8

As experiments 3 and 4 gave very similar results, the growth results from 4 to 14 weeks have been combined and the data obtained tabulated (Table VI) Graphs 3 and 4 give the average growth curves of the male and female rats fed on the 7 different rations —

TABLE VI

*• Protein consumption and growth rate*

Diet	Percentage of protein on dry basis	AVERAGE WEEKLY INCREASE IN WEIGHT (g) OF THE YOUNG RATS DURING 10 WEEKS' EXPERIMENTAL PERIOD	
		Male	Female
B	7.5	3.2	2.0
B + Ca	7.5	3.9	2.3
B + S	13.7	7.7	5.4
B + S + Ca	13.7	7.9	6.1
100B + 12F + S	13.8	12.1	7.9
100B + 12E + S + Ca	13.8	12.2	8.1
100B + 25E + S	13.8	12.9	8.7
100B + 25E + S + Ca	13.8	12.8	8.8
100B + 50E	13.7	12.5	8.5
100B + 50E + Ca	13.7	13.8	9.4
Stock	13.8	13.9	9.6

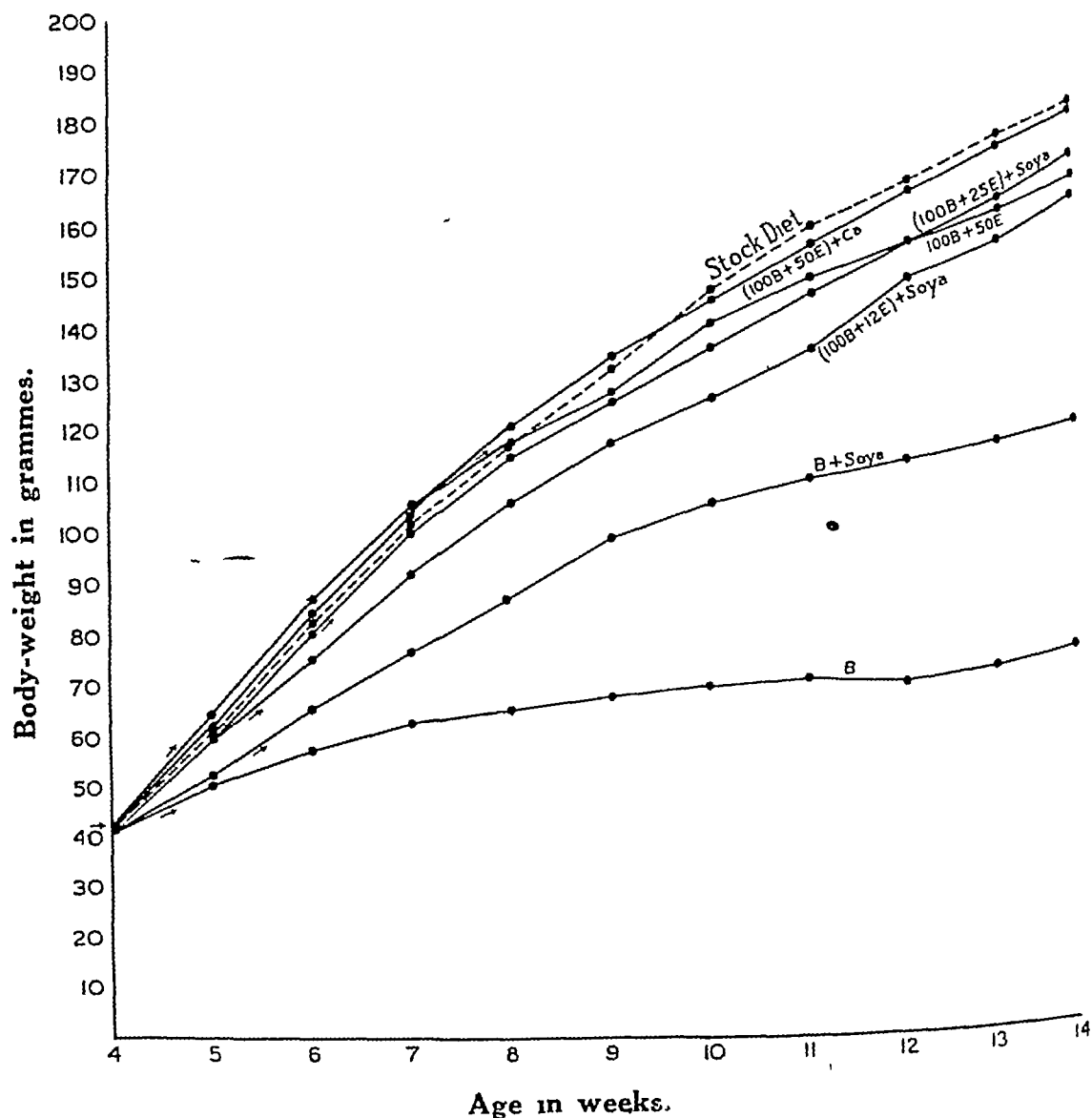


As in experiments 1 and 2, the average weekly gains of the male and female rats from 4 to 14 weeks on the Bengali diet were much below normal. The growth

GRAPH 3.

*Combined growth curves of male rats.*

*(Experiments 3 and 4.)*

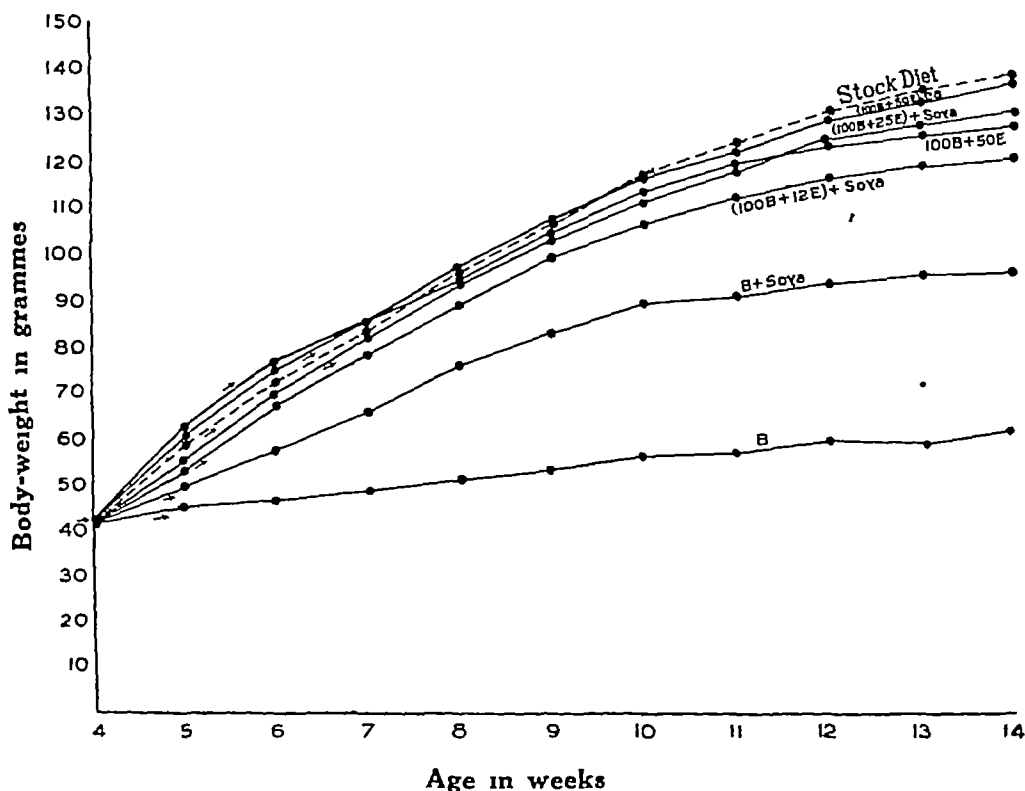


figures for the Bengali diet *plus* egg-shell were again only slightly better than those for the Bengali diet. The addition of soya beans to the Bengali diet resulted in

a marked improvement in the rate of growth. The average weekly gains made by the rats on the Bengali diet *plus* soya beans were, however, very much less than those obtained with the 100B + 50E diet. The male and female rats on the 100B + 25E + S diet made gains in weight similar to those on the 100B + 50E diet. The results obtained with the rats on the 100B + 12E + S diet were

GRAPH 4

*Combined growth curves of female rats*  
(Experiments 3 and 4)



only slightly inferior to those obtained with the diets containing higher proportions of egg. The addition of 0.5 per cent egg-shell to the 100B + 50E diet again proved beneficial. The addition of 0.5 per cent egg-shell did not prove of any material value in the case of any of the rations supplemented with soya beans.

Table VII gives the weight of each ration that would have to be consumed to give a daily intake of 60 g protein. It also gives the number of calories that would be consumed daily and the number of eggs and the weight of the soya beans that would have to be used to supplement the different rations —

TABLE VII

*Calorific value, number of eggs and consumption of soya bean  
to give a daily intake of 60 g protein*

Name of diet	Consumption in g	Calories	Number of eggs required	Soya bean required in g
B + S	536	1,866		85
B + 12E + S	561	1,850	1 2	66
B + 25E + S	612	1,871	2 5	45

Based on Aykroyd's standards for children from 6 to 9 years, the three diets B + S, B + 12E + S and B + 25E + S supply the necessary daily protein of 60 g without an unnecessarily high food consumption.

#### DISCUSSION

In all four experiments with rats, the Bengali diet gave very poor growth results. Moreover, general health was poor and 4 out of a total of 16 rats fed on this diet showed marked swelling of the sub-maxillary glands. Four rats reared on the Bengali diet *plus* egg-shell also showed similar swellings. As none of the rats on the other diets showed glandular enlargement, it is apparent that the Bengali diet was deficient in some factor necessary for the prevention of this condition. Furthermore, as the rats on the Bengali diet from 4 to 20 weeks were typical cases of vitamin A deficiency and as the livers of these rats showed complete absence of vitamin A, it is more than probable that the gland swellings were due to a lack of this vitamin, which is known to be necessary as a safeguard against certain infections. The occurrence of vitamin A deficiency in the rats after a prolonged period on the Bengali diet is of considerable interest in that it corroborates the belief that night-blindness is of very frequent occurrence amongst villagers in Bengal.

With eggs as the sole supplement to the Bengali diet, the best results were obtained with the 100B + 50E diet. However, the 100B + 50E diet was somewhat deficient in calcium and results, equal to those achieved with the control diet, were only obtained by the addition of 0.5 per cent ground egg-shell. Though the 100B + 50E + Ca appears to be very satisfactory from the nutritional standpoint for children, it cannot be regarded as a practical diet for it is most unlikely that

human subjects could be induced to supplement their diet with calcium in the form of egg-shell or other inorganic substances. Furthermore, to satisfy the necessary requirements for growth and maintenance in children from 6 to 9 years, it would be necessary to consume 5.2 eggs daily (internal content 45 g), an amount which would be much too costly for the majority of the population.

In the second series of experiments, the 100B + 25E + S diet gave slightly better final results than the 100B + 50E diet. As the 100B + 25E + S diet only requires to be supplemented with 2.5 eggs and 45 g soya beans daily, this ration is considerably more economical than the 100B + 50E diet. However, though the 100B + 12E + S diet proved somewhat inferior to the 100B + 50E diet, the results may be considered fairly satisfactory for young rats for their general health and condition remained reasonably good throughout. From the human standpoint, the 100B + 12E + S diet may be considered fairly satisfactory from the economic aspect, for the necessary requirements for children from 6 to 9 years can be supplied by the Bengali diet *plus* 1.2 eggs and 66 g of soya beans daily.

### CONCLUSIONS

- 1 Very unsatisfactory growth rates and poor general health were obtained in young rats fed on a typical Bengali village diet.
- 2 The Bengali diet, even when supplemented with calcium in the form of egg-shell, still remained deficient in proteins and vitamins.
- 3 Supplements of egg to the Bengali diet had a very beneficial influence on the rate of growth and general health.
- 4 The addition of calcium in the form of egg-shell to the egg-supplemented Bengali diet improved the growth rate.
- 5 Supplements of soya beans, though beneficial, proved markedly inferior to supplements of eggs.
- 6 Supplements of soya beans *plus* eggs were superior to supplements of soya beans only.
- 7 Addition of calcium in the form of egg-shell to the various soya bean-supplemented diets had no beneficial effect.
- 8 From the purely economic aspect of human nutrition, eggs as the sole protein supplement are too costly.
- 9 From the nutritional aspect, a Bengali village diet in conjunction with a daily supplement of 1.2 eggs and 66 g soya beans appeared to satisfy the dietary requirements of children from 6 to 9 years of age.

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# ESTIMATION OF FOOD VALUE BY CHEMICAL METHODS OF ANOTHER SERIES OF EDIBLES CONSUMED IN BIHAR

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[Received for publication, December 31, 1941]

## INTRODUCTION

IN continuation of investigations which have already been reported (Mitra, 1938, Mitra, Mittra and Roy, 1940, Mitra and Mittra, 1941) on the composition of foodstuffs in use in Bihar, the results of the examination of a further series of 80 items are now presented. These include many not previously examined and along with the results recorded by other workers, including the recent publications of Saha and Ghosh (1941) and Rattan and Sen (1941), will help towards completing tables of food values which are required by those engaged in diet survey and other nutrition work in India.

## EXPERIMENTAL

The technique described in a previous publication by the senior author (Mitra, *loc cit*) has been followed and the analysis of iron has been left out on account of extreme variability of available iron reported in different groups of foodstuffs by Ranganathan (1938). Special precaution was taken to analyse the foodstuffs in as fresh a state as possible. Whenever the foodstuffs were procured from rural areas in distant places within the province they were brought to the laboratory in an ice-chest by one of the laboratory attendants and analysis carried out without the least possible delay.

TABLE.  
Food value of edible portions in grammes per cent.

Serial number	Hindi or local name of the foodstuff	English name	Latin name	Moisture, g	Protein, g	Ether extractives, g	Carbohydrates, g	Mineral matter, g	Calcium, g	Phosphorus, g	Crude fibre, g
1	GRAIN FOODS										
1	Kushi kelao	Field pea	<i>Pisum arvense</i>	10.14	22.03	1.17	57.27	2.29	0.074	0.252	6.10
2	Marha	Common millet (boiled and parched)	<i>Panicum mitaceum</i>	6.54	12.90	1.96	75.76	1.99	0.015	0.351	0.85
3	Marua sattoo	Ragi powder	<i>Eileusine coracana</i>	11.67	4.41	0.79	79.41	2.32	0.182	0.159	1.40
4	Kudrum ke bna	Seeds of roselle	<i>Hibiscus Sabdariffa</i>	6.45	20.86	21.28	31.08	4.88	0.310	0.643	15.45
5	FLESH FOODS										
5	Chahee	The wood sand-piper	<i>Tringa galareola</i>	72.12	22.92	2.10	1.45	1.41	0.008	0.300	
6	Ghonghi	Snail (small)	<i>Viviparus bengalensis f. typica</i> (Lamarck)	78.89	12.63	0.99	3.08	3.81	1.321	0.147	
7	Hau or mata	Red ants with eggs	<i>Ecophylla smaragdina</i> Fab	71.58	13.38	4.60	9.12	1.32	0.104	0.107	
8	Kekra (chhota)	Small crab	<i>Paratelphusa spinigera</i> (W. M.)	65.27	11.15	9.83	9.20	4.55	1.006	0.253	
9	Kocho mnehli (andha sanp)	Amphipnous (Hamilton)	<i>Amphipnous cuchia</i>	76.69	18.73	0.80	2.34	1.44	0.185	0.119	
10	Soor ka gosht	Pork	<i>Sus cristatus</i> W. agner (domesticated)	73.00	21.81	3.47	0.59	1.04	0.013	0.105	

Fruits		Indian pruno (mpe and fresh)	Prunus bokhariensis	85.27	0.50	0.28	12.88	0.00	0.010	0.018	0.47
11	Alubokhar (kaja)										
12	Baincha		<i>Flacourtia Ramonitchii</i> L'herit	67.81	1.09	1.78	22.70	1.34	0.092	0.053	4.68
13	Barh ka pakua	Figs of banyan tree	<i>Ficus bengalensis</i>	74.13	1.74	1.06	11.81	1.00	0.364	0.043	8.46
14	Deshi khajur	Dates (Indian)	<i>Phoenix syriacensis</i>	59.20	1.22	0.42	33.80	1.71	0.022	0.078	3.65
15	Gulab jamun	Rose apple	<i>Eugenia jambos</i>	85.19	0.92	0.69	11.53	0.56	0.026	0.023	1.11
16	Jamrool	Star apple	<i>Eugenia javanica</i>	91.87	0.31	0.24	6.28	0.30	0.005	0.010	1.00
17	Kasaru	Bid root	<i>Scurpus grossus</i> Linn var Kysoor Clarke	47.74	2.07	0.26	17.08	1.07	0.011	0.133	0.88
18	Kusum ka phal	Kusum fruits	<i>Schleichera tryuga</i>	80.24	1.54	0.78	9.83	1.01	0.015	0.042	0.60
19	J ichi ka baoha (ansfal)	Bastard ichi	<i>Nephelium longana</i>	83.85	1.38	0.32	13.19	0.77	0.015	0.035	0.49
20	Mata sara (whole fruit)		<i>Antidesma ghazemballa</i>	72.28	1.88	1.01	10.67	1.05	0.138	0.028	13.11
21	Mata sara (skin and pulp)		" "	84.14	2.27	1.03	9.04	1.19	0.016	0.022	1.73
22	Mulchhari		<i>Minutopsis Elangi</i>	54.71	1.83	0.95	35.86	2.31	0.212	0.030	4.34
23	Nona atwa	Bullock's heart	<i>Anona reticulata</i>	78.78	1.30	1.04	12.72	0.97	0.028	0.024	5.19
24	Pipar ko pakua	Figs of pipal tree	<i>Ficus religiosa</i>	62.42	2.52	1.66	21.25	2.20	0.280	0.080	0.86
25	Saftulu or satulu	Peaches	<i>Prunus persica</i>	83.80	0.67	0.43	13.07	0.85	0.006	0.031	0.58
26	Singhura	Water chest nut	<i>Trapa bispinosa</i>	75.09	3.06	0.24	19.95	1.11	0.020	0.109	0.55
27	Sirka		<i>Zizyphus villosa</i>	55.20	3.24	1.34	33.26	1.99	0.270	0.004	4.88
28	Velva ka topi	Orange calyx of marking nut	<i>Semecarpus anacardium</i>	27.31	2.09	0.03	65.12	2.64	0.237	0.007	2.21
29	Kacheha mahua	Fresh mahua flowers	<i>Bassia latifolia</i>	72.38	1.25	0.20	24.70	0.70	0.026	0.031	0.62



TABLE—contd

Serial number	Hindi or local name of the foodstuff	English name	Latin name	Moisture, g	Protein, g	Ether extractives, g	Carbohydrates, g	Mineral matter, g	Calcium, g	Phosphorus, g	Crude fibre, g
30	Fruits—contd										1 00
	Koril	Tender bamboo shoots (fermented)	<i>Dendrocalamus strictus</i>	94.25	1.81	0.36	1.86	0.72	0.020	0.029	
31	Ukh ka guri	Cane guri	<i>Saccharum officinarum</i>	8.74	0.56	0.10	89.00	1.60	0.063	0.101	
32	Ukh ka ras	Cane juice	" "	82.51	0.37	0.03	16.69	0.40	0.020	0.015	
	VEGETABLES (LEAFY TYPE)										
33	Alti or arui sag		<i>Colocasia antiquorum</i>	84.18	3.44	1.57	7.06	1.79	0.515	0.043	1.96
34	Belagori sag	Wild mustard	<i>Cleome viscosa</i>	83.02	3.30	1.17	8.46	2.30	0.231	0.086	1.75
35	Chakwar or Chakonda sag	Fetid cassia	<i>Cassia tora</i>	84.93	5.02	0.84	5.41	1.68	0.417	0.054	2.12
36	Chura sag	Purslane	<i>Portulaca oleracea</i>	91.02	0.52	0.45	4.77	1.73	0.085	0.026	1.51
37	Gendhri sag	Love lies bleeding	<i>Amarantus caudatus</i>	90.02	2.99	0.66	3.25	2.05	0.196	0.044	1.03
38	Gogta sag		<i>Amarantus gangeticus</i>	85.35	3.41	0.38	6.32	3.13	0.311	0.074	1.41
39	Guloharni		<i>Calonyction muricatum</i>	91.71	1.31	0.52	4.24	1.21	0.059	0.060	1.01
40	Imli (lupu)	Dried tender tamarind shoots	<i>Tamarindus indica</i>	8.91	8.64	3.00	60.87	8.48	1.485	0.124	10.10

41	Katha sag	Water fern	<i>Dentella repens</i>	90 02	1 00	0 47	3 02	2 33	0 253	0 035	1 06
42	Konn sag	" "	<i>Commelina benghalensis</i>	92 21	2 13	0 30	2 50	1 97	0 070	0 047	0 80
43	Khapra sag (nakli gat-purna)	Horse purslane	<i>Trianthema monogyna</i>	92 10	2 50	0 48	1 87	2 20	0 055	0 040	0 85
44	Kohra ka sag	Pumpkin leaves	<i>Cucurbita mazima</i>	81 80	4 59	0 82	7 80	2 72	0 392	0 112	2 12
45	Lauka sag	Leaves and stem of bottle gourd	<i>Lagenaria vulgaris</i>	87 02	2 33	0 09	6 00	1 71	0 080	0 059	1 20
46	Mata sag (lupu)		<i>Andesma danthrum</i>	7 21	7 16	4 78	57 80	9 40	1 717	0 080	13 50
47	Kudrum sag	Roselle leaves	<i>Hibiscus Sabdariffa</i>	82 02	3 50	0 08	10 10	1 32	0 200	0 062	1 03
48	Patua sag	Jute plant	<i>Cochorus olitorius</i>	83 00	0 01	1 01	4 70	1 80	0 237	0 090	2 82
49	Piyaz ka patli	Onion tops	<i>Allium cepa</i>	90 25	1 10	0 75	5 42	1 00	0 078	0 014	1 42
50	Saijan ka phool (munga phool)	Drumstick flowers	<i>Moringa oleifera</i>	85 85	3 57	0 82	7 10	1 41	0 081	0 090	1 25
51	Sanni ko phool	San hemp flower	<i>Crotalaria juncea</i>	78 88	4 77	0 04	10 50	1 35	0 167	0 075	3 80
52	Saro sag (lupu)	Dried colocasia leaves	<i>Colocasia antiquorum</i>	9 31	13 72	5 93	42 25	12 79	1 540	0 308	10 00
53	Satiso sag (lupu)	Dried rapo leaves	<i>Brassica napus</i>	7 30	26 00	2 88	40 70	15 34	3 095	0 500	6 70
54	Sarniti sag		<i>Alternanthera sessilis</i>	85 44	4 12	0 73	4 50	3 48	0 312	0 058	1 64
55	Semal ka phool	Tender flowers of silk cotton	<i>Bombax malabaricum</i>	86 44	1 50	0 30	9 47	0 73	0 022	0 045	1 50
56	Sinduar sag (rural)		<i>Celosia argentea</i>	88 03	1 05	0 06	5 80	2 04	0 323	0 038	1 52
57	Sinduar sag (jungle)		<i>Althausia nodiflora</i>	87 43	1 10	0 80	4 38	2 60	0 104	0 040	3 09
58	Suani sag	Water fern	<i>Marsilia quadrifolia</i>	80 04	3 72	1 38	4 61	2 06	0 063	0 001	1 29
59	Utarba (jirhoal ka phool)		<i>Indigofera pulchella</i> var <i>purpurascens</i>	85 91	4 24	0 00	5 50	1 73	0 254	0 078	1 90

TABLE—*concl'd*

Serial number	Hindi or local name of the foodstuff	English name	Latin name	Moisture, g	Protein, g	Ether extractives, g	Carbohydrates, g	Mineral matter, g	Calcium, g	Phosphorus, g	Crude fibre, g
	VEGETABLES (ROOTS AND TUBERS)										
60	Amathi (amahaldi)	Sweet ginger	<i>Curcuma amata</i>	79.72	1.05	0.90	15.40	1.59	0.034	0.153	1.34
61	Bola or paika		<i>Dioscorea bulbifera</i>	65.22	4.73	0.12	27.40	1.33	0.013	0.109	1.20
62	Bokwa or kulu		<i>Dioscorea pentaphylla</i>	79.62	2.85	0.31	15.55	0.79	0.025	0.063	0.88
63	Badhua		<i>Melothria heterophylla</i>	66.52	0.68	0.36	29.96	0.86	0.019	0.043	1.62
64	Chumbia		<i>Dioscorea Hamiltonii</i>	66.65	1.78	0.17	28.94	0.98	0.052	0.049	1.46
65	Churkia or baiyang		<i>Dioscorea glabra</i>	83.66	1.63	0.10	12.77	0.61	0.010	0.038	1.23
66	Moor sangra (dhak)		<i>Butea frondosa</i>	45.43	2.10	0.25	50.01	0.62	0.025	0.021	1.59
67	Sang		<i>Dioscorea anguina</i>	65.03	2.26	0.08	30.89	1.01	0.039	0.064	0.73
68	Turum sangra		<i>Curculigo orchitoides</i>	66.40	1.85	0.35	28.12	1.71	0.342	0.010	1.57

60	Tigo or haser	<i>Dioscorea pentaphylla</i> var <i>communis</i> Burkhill	85 84	2 07	0 12	9 94	0 60	0 023	0 042	0 83
70	Uaungid	<i>Hibiscus cancellatus</i>	80 04	1 90	0 02	10 93	2 57	0 520	0 042	3 64
71	Chichura	Snake gourd <i>Trichosanthes anguina</i>	90 19	0 83	0 10	1 52	0 40	0 025	0 016	0 87
72	Karonda	<i>Carsaea carandus</i>	90 95	1 08	2 89	2 94	0 60	0 021	0 028	1 54
73	Lama ko biyaka ghudda	<i>Bauhinia vahlii</i>	25 47	23 72	28 15	17 34	3 02	0 284	0 008	2 30
74	Mural ko dhondhi	Green pods from radish tops <i>Raphanus sativus</i>	89 40	1 88	0 56	5 00	0 95	0 152	0 066	1 22
75	Parha (gullar)	Red figs <i>Ficus cumia</i>	79 35	1 23	0 04	10 83	1 57	0 187	0 039	6 38
76	Sang ka phal	<i>Dioscorea anguina</i>	73 44	1 48	0 13	23 06	0 87	0 016	0 048	1 02
77	Bhais ka dudh	Buttermilk (buffalo)	79 74	4 39	9 45	5 70	0 72	0 259	0 188	
78	Cher ka dudh	Goat milk	85 20	3 71	5 60	4 69	0 80	0 134	0 103	
79	Gal ka dudh	Cow milk	87 93	3 30	3 80	4 26	0 71	0 123	0 086	
80	Metha (bhais)	Buttermilk (buffalo)	91 15	2 63	1 21	4 31	0 68	0 162	0 097	

## DISCUSSION

*Grain foods* —Amongst the grain foods analysed the seeds of roselle deserve special mention as being rich in protein and mineral matter. The seeds are baked over hot sand and consumed raw with other parched grains by the comparatively poorer sections of the population.

*Flesh foods* —Ghonghi, a small variety of snail, is very rich in calcium and being poor in fat is often prescribed by village doctors for children with delicate stomachs. It is consumed mostly by people of the lower social strata. Red ants with eggs are a favourite food of aboriginal children and are consumed in raw state (at times baked over a fire). These are sold in almost all the 'hatias' (weekly markets) in Chotanagpur and there is a great demand for them.

*Fruits* —Bastard lichis are not grown in this province and the sample was procured from Calcutta. Except gulab jamun, jamrool, fresh alubokhara and saftalu, all the other fruits included in the Table are sold very cheaply or else are available in plenty in orchards and jungles within the province. Fruits comparatively rich in calcium are banyan figs, mulchhari (fruits of bakul tree), figs of pipal tree, sirka and the orange calyx of marking nut.

*Leafy vegetables* —Amongst the fresh leafy vegetables analysed, arui sag (colocasia leaves) was found to be the richest source of calcium (515 mg/100 g), whereas khapra sag gave the lowest figure (55 mg/100 g). The aboriginal and semi-aboriginal people in Chotanagpur dry leafy vegetables (lupu) and consume them in seasons of scarcity.

*Root vegetables* —Of the eleven kinds of tubers analysed usungid (*Hibiscus cancellatus*) and turum sanga (*Curculigo orchroides* Gaertn.) were found to be unusually rich in calcium.

*Other vegetables* —The seeds of lama pods are rich in fat and to a certain extent in mineral matter. The pods of radish also deserve mention on account of their high calcium content. The various tubers analysed are consumed very largely by the different aboriginal tribes in Chotanagpur during seasons of scarcity.

## SUMMARY

Four kinds of grain foods, 6 of flesh foods, 22 of fruits, 27 of leafy vegetables, 11 of roots and tubers (vegetables), 6 of other vegetables and 4 of milk and milk products have been chemically analysed to determine their respective food values.

## ACKNOWLEDGMENTS

The authors are grateful to Dr. Baini Prasad, Director, Zoological Survey of India, Calcutta, to Professor S. P. Agharkar, Head of the Department of Botany, Calcutta University, and to Professor S. S. Chaudhury, Head of the Department of Biology, Prince of Wales Medical College, Patna, for all the assistance rendered in compiling the Latin names, to Babu Jwala Prasad Pandey, Headmaster,

Jagannathpur H E School, for his valuable help in collecting specimens of edibles from the Singhbhum district and finally to Lieut-Colonel S L Mitra, I M S, and Rai Bahadur Dr B P Mozoomdar, successive Directors of Public Health, Bihar, for their interest in the work

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## STUDIES IN HUMAN NUTRITION

### Part IV

#### AVAILABILITY OF CALCIUM INGESTED IN THE PROCESS OF CHEWING BETEL-LEAVES WITH LIME

BY

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[Received for publication, December 31, 1941]

METABOLIC studies on human beings carried out in this Laboratory (Basu *et al*, 1941) have shown that Indian dietaries without milk are extremely deficient in calcium, and that this deficiency can be removed by supplementing them with at least 10 oz of milk per day. Milk and milk products are by far the richest, but at the same time the most costly, sources of food calcium. The consumption of dairy products in any country depends upon the economic condition of the people (Orr, 1937, Wilson *et al*, 1936, 1937, 1938) and increases or decreases almost directly with the level of income. The question of the provision of an adequate quantity of calcium in dietaries is thus much more important in the case of the poor than anybody else, its acuteness increasing with that of poverty. In view of the poverty of the large majority of the population in India, where *per capita* production and consumption of milk is only 7 oz per day as compared with 35 oz in many other countries (Wright, 1937) it is essential that, besides encouraging the production of more milk, some very cheap but rich sources of available calcium



should be sought which may be a substitute for milk as far as the requirement of calcium is concerned

Aykroyd and Krishnan (1937) suggested the use of calcium lactate. We on the other hand have been on the look out for foodstuffs rich in calcium and also for food habits which would ensure the intake of sufficient available calcium at a very little cost. It was thought that the habit of chewing betel-leaves with lime, betel-nuts and catechu after meals—a very popular habit in India, specially among rural populations—might provide a fair amount of available calcium. A good amount of calcium is ingested in this process and it costs only about 2 to 4 pies daily for a family of six. Aykroyd (1937) and Wilson and Mitra (1938) commented on this habit and pointed out that it increases the intake of calcium. But nothing is known as yet about the utilization of calcium from this source. Aykroyd (*loc cit*) remarked that 'at present we have no precise knowledge of the value to the body of calcium consumed in this manner'. The aim of the present investigation was to study the absorption and utilization of calcium ingested in the process of chewing betel-leaves with lime and other ingredients by direct metabolic experiments on human subjects.

#### EXPERIMENTAL

Experiments were conducted on five young healthy adults, of ages ranging between 20 and 34 years and body-weights between 45 and 60 kilos. They were fed on typical rice or wheat diets whose composition has been described in a previous communication (Basu *et al*, 1941) for six days. The chewing of betel-leaves with lime, betel-nuts and catechu after meals was then prescribed and the experiments were continued for another six days. Each period included three consecutive days and only the mean of the daily analytical figures for each period is presented in Tables I and II. The chronological order of the experimental periods is denoted by arithmetical numbers and the periods which do not figure in the table must be assumed to be the preliminary periods. Technique and analytical methods were similar to those used in previous investigations from this Laboratory (Basu *et al*, 1939).

TABLE I

#### *Metabolism on rice diet*

Period	Dietary Ca per day (mg)	Dietary Ca/P	Urinary Ca per day (mg)	Faecal Ca per day (mg)
P-2	200	1/8.4	17	194
P-4	390	1/4.3	19	200

TABLE I—concl'd

Subject	Diet	Period	Dietary Ca per day (mg)	Dietary Ca/P	Urinary Ca per day (mg)	Faecal Ca per day (mg)	Balance (mg)
P C D	Rice fish diet	P 2	310	1/5 9	26	510	-226
	Rice fish diet + 6 betel leaves, lime, etc	P 3	656		78	700	-122
	Do	P-4	627		70	538	+19
	Do	Average of P 3 and P 4	642	1/2 8	74	619	-51
S G P	Rice fish diet	P 2	299	1/4 7	96	253	-50
	Rice fish diet + 6 betel leaves, lime, etc	P 3	589		160	346	+83
	Do	P-4	776		181	348	+247
	Do	Average of P 3 and P-4	683	1/2 1	171	347	+165

TABLE II

*Metabolism on whole-wheat (atta) diet*

Subject	Diet	Period	Dietary Ca per day (mg)	Dietary Ca/P	Urinary Ca per day (mg)	Faecal Ca per day (mg)	Balance (mg)
P C D	Wheat diet	P 2	380	1/4 6	25	410	-55
	Wheat diet + 6 betel- leaves, lime etc	P 3	656		40	514	+104
	Do	P-4	755		46	624	+85
	Do	Average of P-3 and P-4	707	1/2 5	43	569	+95

TABLE II—concl'd

Subject	Diet	Period	Dietary Ca per day (mg)	Dietary Ca/P	Urinary Ca per day (mg)	Faecal Ca per day (mg)	Balance (mg)
P C G	Wheat diet	P-2	415	1/4 7	33	440	-58
	Wheat diet + 6 betel- leaves, lime, etc	P 3	819		72	300	+447
	Do	P 4	805	1/2 4	71	606	+128
	Do	Average of P 3 and P-4	812		72	453	+287
G C D	Wheat diet	P 2	340		14	321	+ 5
	Wheat diet + 6 betel- leaves, lime, etc	P-3	710		31	600	+79
	Do	P 4	801		33	536	+232
	Do	Average of P-3 and P-4	755		32	568	+155

## DISCUSSION

The results show that calcium ingested in the process of betel-leaf chewing with lime etc, not only increased the intake of calcium and made the ratio of calcium to phosphorus in the diet more favourable for absorption and utilization, but was also retained by the subjects to a considerable extent. In fact, if the metabolic data recorded here are compared with those in the preceding paper (Basu *et al*, 1941), it will be found that the amount and the utilization of calcium ingested with 6 betel-leaves was similar to that from 10 oz of cow's milk. Hence, it can be concluded that calcium from this source is a very cheap substitute for milk calcium.

The metabolism of phosphorus was also studied along with that of calcium. The subjects were always in positive phosphorus balance which was not disturbed by the administration of extra calcium with betel-leaves.

## SUMMARY

Calcium ingested in the process of chewing of betel-leaves with lime, betel-nuts and catechu—a very popular habit in India—is well absorbed and utilized, and thus can well meet the deficiency of calcium in normal dietaries

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# INVESTIGATIONS ON THE FOOD VALUE OF FISH AND OTHER MARINE PRODUCTS

## Part I

### THE ANTIPELLAGRA VITAMIN (NICOTINIC ACID)

BY

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[Received for publication, December 31, 1941 ]

FISH constitutes a very important source of food. Although some important work was carried out in India on the nutritive value of fish with respect to the vitamin A content of liver oils, and their content of minerals and proteins, very little work has been reported regarding the nicotinic-acid (antipellagra vitamin) content of fish except that of Saha (1941) on Bengal fish which was published recently during the course of our work. No systematic investigation has, however, been made on the food value of fish available in coastal waters of Waltair. The work reported in this paper was undertaken as a part of a detailed inquiry on the food value of fish which constitute a very important source of food in the Northern Circars. The present paper relates to the nicotinic-acid content of the muscle tissue of various species of fish and other marine products.

## EXPERIMENTAL

*Collection of material*—The fish were obtained fresh from the Government Fish Curing Yard, Lawson's Bay, and the muscle tissue which forms the edible

portion of the fish was removed and finely minced. The fine tissue pulp was directly used for the determination of nicotinic acid.

*Method of estimation of nicotinic acid*—The method for the determination of the nicotinic-acid content followed in the present investigation was the adsorption method developed by Giri and Naganna (1941a, b).

Ten grammes of the finely minced fish-muscle tissues were extracted with water as described before (Giri and Naganna, 1941b) and the total volume of the extract, which amounted to about 50 ml to 60 ml, was subjected to hydrolysis with sodium hydroxide in order to convert the nicotinamide present in the extract into nicotinic acid. In the case of some fish, which contain more fat in their muscle and liver, clear extracts can be obtained by keeping in the refrigerator for some time before centrifuging. The hydrolysed extract was then neutralized and the nicotinic acid was adsorbed on medicinal charcoal. The charcoal adsorbate was eluted with alcohol-sodium hydroxide mixture and the eluate was neutralized and adjusted to pH 5.6. A perfectly colourless extract was obtained by this procedure. The total volume of the eluate was always kept within 25 ml. Five ml or 10 ml of the eluate was taken in a 25-ml glass-stoppered graduated cylinder and 5 ml of M/5 acetate buffer (pH 5.6) were added, and the colour produced by adding the reagents cyanogen bromide and p-aminoacetophenone according to the procedure described previously (Giri and Naganna, 1941b), was measured colourimetrically against a standard similarly treated.

Occasionally turbidity develops when the buffer is added to the eluate, and this may be a source of serious error. Turbidity can be easily removed or prevented either by adding a few drops of absolute alcohol until it disappears, or by taking 10 ml of the eluate instead of 5 ml for estimation.

The Table summarizes data which have been obtained on a number of food fishes. The values given are the average obtained by two estimations —

TABLE

*The nicotinic acid content of fish*

Number	Common name	Local name	Zoological name	Nicotinic acid, mg per 100 g of fresh muscle tissue
1	Seer	Vanjaram	<i>Scomberomorus</i> Spp	1.2
2	Shark	Sorra		2.5
3	Jew fish	Gorasalu	<i>Scænidæ</i>	0.63
4	Horse mackerel	Para	<i>Caranx</i> Spp	2.9
5	Ribbon fish	Savallu	<i>Trichiurus haumela</i>	2.1

TABLE—concl'd

Number	Common name	Local name	Zoological name	Nicotinic acid, mg per 100 g of fresh muscle tissue
6	Cat fish	Jellalu	<i>Siluridae</i>	2.5
7	—	Golvmdalu		2.3
8	—	Barai Matta	<i>Saurida tumbil</i>	2.9
9	Hilsa	Pulasalu	<i>Hilsa ilisha</i>	4.7
10	Pomfrets	Chanduva	<i>Stromateus</i> Spp	2.6
11	Silver bellies	Karalu	<i>Leiognathus</i> Spp	2.9
12	White bait	Nethallu	<i>Stolephorus</i> Spp	2.3
13	Mulletts	Bonthalu	<i>Mugil</i> Spp	2.6
14	Sabre fish	Mulluvalava	<i>Chirocentrus dorab</i>	3.5
15	Pollona	Engallu	<i>Pellona</i> Spp	3.6
16	Sardines	Kavallu	<i>Sardinella fimbriata</i>	2.6
17	—	Sudumullu		2.2
18	Prawns	Royyalu		4.8
19	Crabs	Peethalu		3.1
20	Cuttle fish	Kalimbda	<i>Sepia</i> Spp	2.9
				Mg per 100 g of fresh liver
	Seer	Vanjaram	<i>Scomberomorus</i> Spp	3.4
	Shark	Sorra		3.2

## DISCUSSION

A comparison of this Table of results with one dealing with the common fish of Bengal (Saha, *loc cit*) shows that the nicotinic acid content of Bengal fish is considerably lower than the values obtained in the present investigation. Saha reported values ranging from 0.32 mg to 1.02 mg per 100 g of fresh fish-muscle tissue, and among the 20 fish analysed, all except two contained less than 1.0 mg nicotinic acid per 100 g of fresh tissue. It is thus clear from the results obtained on Bengal fish that they are poor sources of the vitamin. On the other hand, Kodicek (1910) from Cambridge reported higher values for the fish



investigated salmon 8.4 mg, herring 4.0 mg and cod 3.0 mg, per 100 g of tissue, and Leong (1940) from Singapore reported that the nicotinic-acid content of salmon was about the same as that of meat and that two other species of fish available in Singapore were found to give much lower values (aryan 0.6 mg and pomfret 0.9 mg per 100 g)

We are led to infer from our investigation that the value for the nicotinic acid content of the majority of fish investigated is of the same order as that of mammalian muscle tissue (Giri and Naganna, 1941b). Except in two cases, the values obtained for the fish were always greater than 2.0 mg per 100 g. The low values obtained by Saha (*loc cit*) may be attributed to the fact that the fish analysed in Bengal were mainly fresh-water fish. The nicotinic-acid content of the liver of fish is not as great as that of the livers of animals. Another interesting point emerging out of the present investigation is that prawns and crabs are good sources of the vitamin, which can be compared to beef with regard to their vitamin potency.

Since fish is a comparatively cheap article of a diet than meat, eggs and milk, particularly in coastal towns like Vizagapatam and Waltair, it constitutes a cheaper source of the vitamin for the poorer classes. A free use of fish and prawns in the diet, therefore, contributes materially to the nicotinic-acid requirements.

#### SUMMARY.

The nicotinic-acid content of a large number of economically important food fishes in the Northern Circars has been determined by the adsorption method described by Giri and Naganna. The nicotinic-acid content of the majority of fishes ranged from 2 mg to 4 mg per 100 g of the muscle tissue. The fish are, therefore, as good sources of the vitamin as beef and other mammalian muscle tissue. Prawns and crabs are also good sources of the vitamin.

#### ACKNOWLEDGMENTS

The authors are grateful to Mr A. Somayaji and Mr S. Varadarajan, Inspectors of Fisheries, Vizagapatam, for the assistance rendered in compiling the correct Latin names, and for arranging to supply the fish for these investigations.

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# ALKALOIDS OF *RAUWOLFIA SERPENTINA* A COMPARATIVE STUDY OF THEIR PHARMACOLOGICAL ACTION AND THEIR RÔLE IN EXPERIMENTAL HYPERTENSION

BY

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## INTRODUCTION

SIDDIQUI and SIDDIQUI (1931), working on the chemistry of *Rauwolfia serpentina* Benth, isolated an alkaloid from the root known as ajmaline Chopra Gupta and Mukherjee (1933), while studying the general pharmacological action of this alkaloid and of the alcoholic extract from *R serpentina* found hypotensive and sedative properties in them Since then, much clinical data have been collected which justify the use of the extract in essential hypertension Later on, two more alkaloids, namely serpentine and serpentinine were isolated by Siddiqui and Siddiqui (1932) and also in the Department of Chemistry of this Institution A preliminary study of the relative toxicity and pressor effects of these three alkaloids was conducted by Chopra and Chakravarti (1941) A comparative study of the pharmacological actions of the three active principles so far isolated as well as the action of the total alkaloids has been attempted in this paper The mechanism of action in each case has also been investigated

## EXPERIMENTAL

In all experiments hydrochloride salts of the alkaloids, which are easily soluble in water, were used

*General pharmacology*

1 *Carotid pressure*—Ajmaline, in doses of 1 mg/kg to 2 mg/kg body-weight, usually produced a slight rise in blood pressure (10 mm to 16 mm Hg). Smaller doses were without any effect and larger doses occasionally produced a moderate fall of blood pressure (not exceeding 10 mm Hg). Serpentinine like ajmaline was also found to bring about a rise in carotid pressure. Serpentine, total alkaloids and the crude extract of *Rauwolfia*, however in identical doses, produced a definite fall. Plate X, figs 1 to 4 show the results obtained with ajmaline, serpentine, serpentinine and total alkaloids on carotid blood pressure in chloralosed cats. The maximum effect was observed with serpentine, where a fall of 30 mm to 40 mm Hg was frequently noticed. The total alkaloids, in identical dosage, were less active than serpentine and its action was also less persistent. A mixture of ajmaline, serpentine and serpentinine in proportions obtainable in their natural state in the root (0.1, 0.08 and 0.008 per cent respectively) also lowered the carotid pressure, but the hydrochloride of the total alkaloids, from which ajmaline, serpentine and serpentinine were removed, was found to be devoid of any pressor activity.

2 *Myocardiography*—Myocardiographic tracings of cat's heart *in situ* revealed that ajmaline and serpentinine in 2 mg/kg doses depressed the amplitude of the auricular and ventricular contractions. Serpentine and the total alkaloids, on the other hand, produced a definite stimulation.

3 *Isolated heart perfusion*—With a concentration of 1 in 200,000 the kitten heart was stimulated by serpentine, serpentinine and the total alkaloids, ajmaline, on the other hand, depressed this organ.

4 *Intra-tracheal pressure*—In 2 mg/kg doses ajmaline, serpentinine and serpentine increased the amplitude of the respiratory movements. This was soon followed by some depression but the rhythm remained unaffected.

5 *Organ volume*—(a) *Spleen* Alteration in the spleen volume was found to be intimately related to the blood pressure changes in cats. Splenic dilatation was observed when there was a fall of carotid pressure from serpentine and the total alkaloids. A contraction of the spleen volume was noticed with the pressor effects of ajmaline and serpentinine. (b) *Gut* Serpentine and the total alkaloids increased its volume, ajmaline and serpentinine increased its contractions.

6 *Isolated guinea-pig and kitten intestine*—Ajmaline in 1 in 100,000 concentration, increased the peristaltic movements of the intestine. In 1 in 50,000 and 1 in 25,000 concentration sudden tonic contraction of the intestine with temporary cessation of peristalsis occurred. Serpentine and the total alkaloids, in similar doses produced relaxation while serpentinine produced tonic contraction of the organ (Plate X figs 5 to 8).

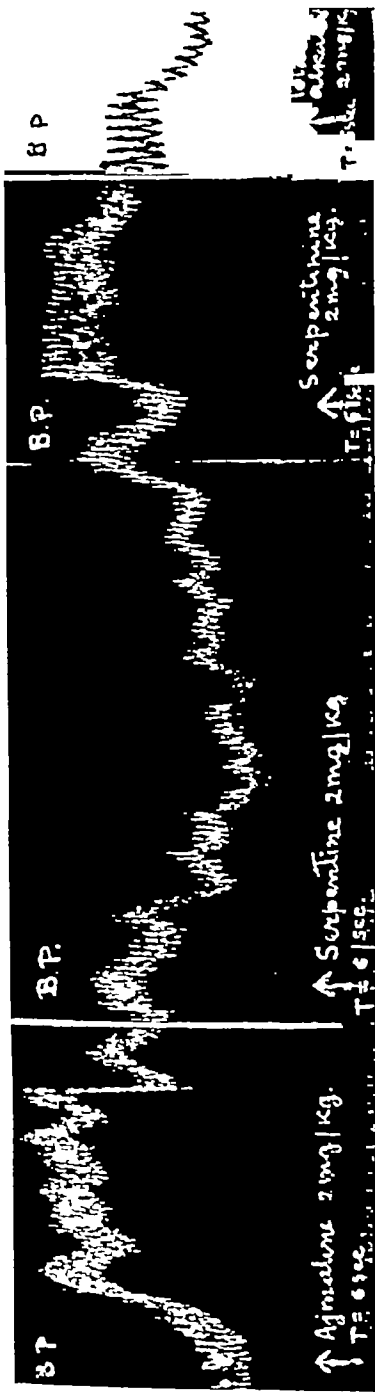


Fig 1.

Fig 2.

Fig 3.

Fig 4.

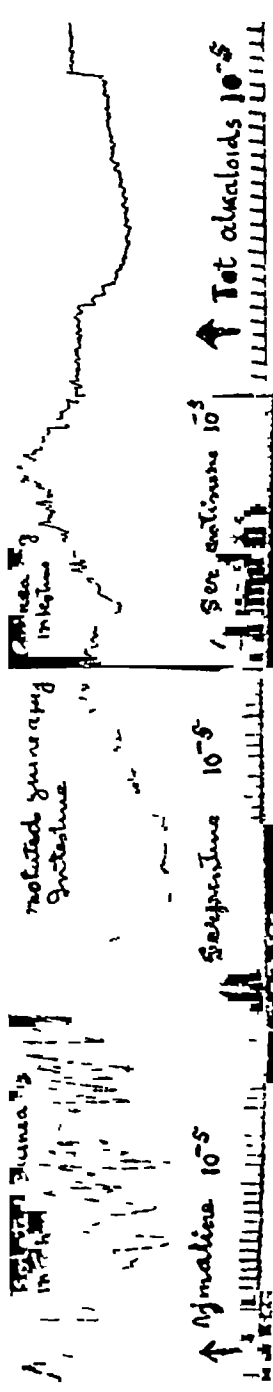


Fig 5.

Fig 6.

Fig 7.

Fig 8.

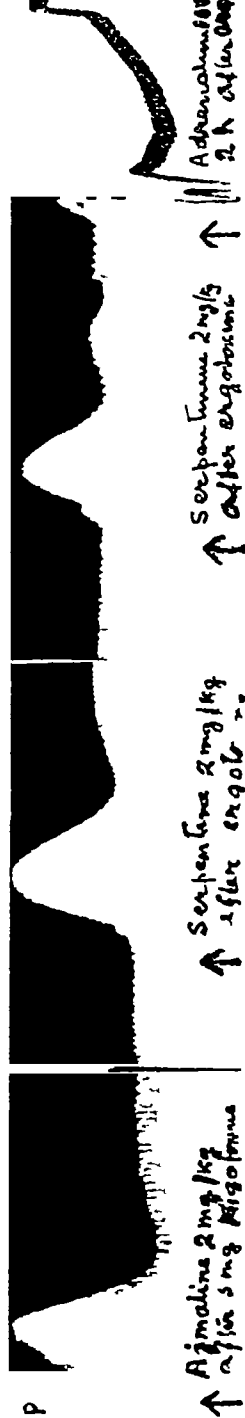


Fig 9.

Fig 10.

Fig 11.

Fig 12.

# Action of Rauwolfia serpentina alkaloids

- Figs 1 to 4 — Action on the carotid pressure of cat
- Figs 5 to 8 — Action on cat's intestine *in situ* and isolated guinea pig intestine
- Figs 9 to 12 — Action of ajmaline, serpentina, serpentinine and adrenalin on the cat's blood pressure after paralyzing the sympathetic nerve endings with ergotoxin

Vaso motor reversal e pressure =  
with ephedrine

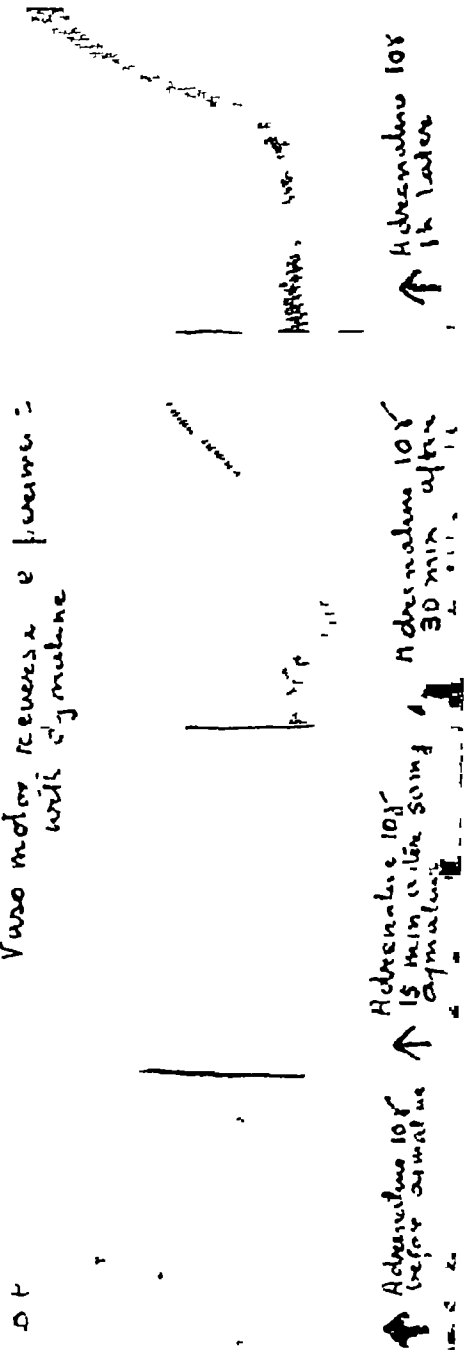


FIG. 1.

FIG. 2.

FIG. 3.

FIG. 4.

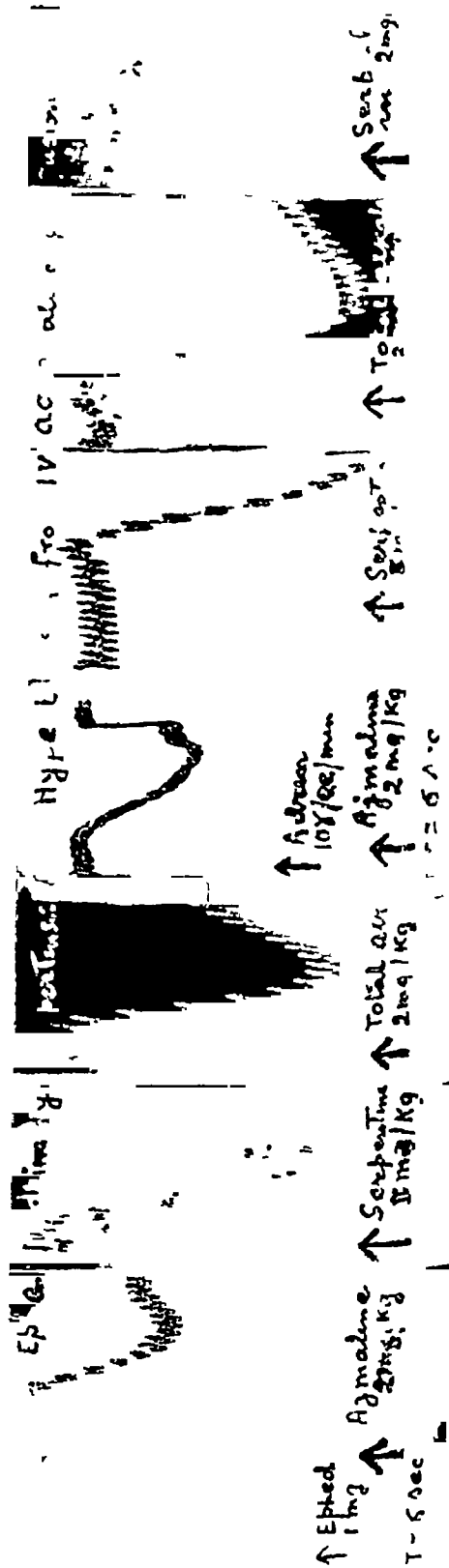


FIG. 5

FIG. 6

FIG. 7

FIG. 8

FIG. 9

FIG. 10

FIG. 11

Action of Rauwolfia serpentina alkaloids.

Figs 1 to 4 — Vaso motor reversal effect of ajmaline

Figs 5 to 11 — Action of the Rauwolfia alkaloids in induced hypertension with ephedrine and adrenalin

## INVESTIGATIONS ON THE MECHANISM OF ACTION

1 *Comparison of the pressor effect in normal, spinal and decerebrate cats* — From what has been already stated, it would be seen that ajmaline and serpentine in moderate doses increased the carotid pressure but ajmaline depressed it in higher concentrations. Serpentine, total alkaloids and the alcoholic extract were pressure depressants. In spinal cats, ajmaline, serpentine and the total alkaloids depressed and serpentine either stimulated or depressed the pressure. In decerebrate animals ajmaline and serpentine stimulated but serpentine and the total alkaloids depressed the carotid pressure.

2 *Effect of atropinization* — Paralysis of the vagal endings, obtained with 15 mg/kg to 2 mg/kg of atropine sulphate and tested against acetyl-choline or the faradic stimulation of the vagus, showed that the pressor effect of *R serpentina* was not very much modified by atropine excepting that ajmaline was found to lose its hypertensive properties in some experiments. On isolated strips of guinea-pig intestine complete atropinization controlled by pilocarpine did not alter the normal action of *Rauwolfia* alkaloids.

3 *Effect of ergotoxine* — The sympathetic nerve-endings were paralysed by the slow intravenous injection of 5 mg of ergotoxine and tested against adrenalin (Plate X, fig 12). The action of ajmaline, serpentine and serpentine was studied in such preparations (Plate X, figs 9 to 12). It was noticed that in this condition the pressor effect of ajmaline was not only quantitatively reduced but even moderately reversed. The action of serpentine was almost unaffected and the normal hypotensive property of serpentine was substituted by a definite hypertension.

4 *Vasomotor reversal effect of ajmaline* — Certain resemblances between the pressor effect of ajmaline and ergotoxine made us investigate if a heavy dose of ajmaline could not paralyse the sympathetic nerve-endings. Accordingly, 50 mg of ajmaline were injected in divided doses, through the femoral vein of normal cats and the effect of adrenalin studied thereafter. Plate XI, figs 1 to 4 show that the adrenalin effect is temporarily reversed by ajmaline and the latent period of action prolonged. This effect passed off after an hour. It was thus evident that ajmaline could temporarily produce a partial reversal of the vasomotor system.

## RELATIVE VALUE OF *R serpentina* ALKALOIDS IN EXPERIMENTAL HYPERTENSES

In view of the wide clinical application of this drug in hypertension, it was considered desirable to study the effect of the individual alkaloids on experimentally induced hypertension brought about by the continuous intravenous perfusion of adrenalin or by the injection of ephedrine. This was expected to throw further light not only on the intensity of action, in this regard of the various active ingredients of the drug, but would also afford the opportunity of a comparative evaluation of the different alkaloids in experimental therapeutics.

1 *Hypertension induced by intravenous adrenalin and ephedrine perfusion* — After recording the action of a definite dose of ajmaline, serpentine serpentine

and the total alkaloids, adrenalin perfusion through the femoral vein was begun. The rate of flow of adrenalin solution was regulated with great precision to 10% or 20% per c c per minute throughout the duration of the experiment for obtaining a constant sub-maximal effect. In case of ephedrine, a single dose of 1 mg of the drug was injected through the femoral vein. When the pressure level was more or less constant, identical doses of the alkaloids were injected as before. The results are summarized in the Table —

TABLE

*Effect of Rauwolfia serpentina alkaloids in adrenalin and ephedrine hypertension*

Name of alkaloid	Dose, mg / kg	Pressor effect of the alkaloids in normal cats, mm Hg	PRESSOR EFFECT IN HYPERTENSIVE CATS		Initial B P level, mm Hg	B P LEVEL AFTER	
			Adrenalin perfusion fall, mm Hg	Ephedrine injection fall, mm Hg		Adrenalin	Ephedrine
Ajmaline	2	16 (rise)	40	40	90	160	162
Serpentine	1	30 (fall)	80	80	60	164	164
Serpentinine	2	10 (rise)	36	26	90	150	122
Total alkaloids	2	20 (fall)	70	76	100	154	158

It is evident from the Table and from Plate XI, figs 5 to 11, that in experimental hyperpiesis in cats, *Rauwolfia* alkaloids produced universal hypotension and this was true even for ajmaline and serpentinine which normally raised the carotid pressure. Maximum reduction was, however, obtained with serpentine and the total alkaloids in which cases the fall of carotid pressure was almost thrice as great as before adrenalin and ephedrine. Ajmaline and serpentinine action was found to be inferior to the above two.

2 *Hypertension produced by paralysing the carotid sinus control* — Both the sinuses were dissected out in cat by the usual technique of sinus dissection. The bulbous portions were searched for at the bifurcation of the common carotid arteries. The dissection was continued up to the root of the internal carotids. These were carefully dissected and swabbed with 5 per cent phenol till the innervations in the adventitia were completely paralysed (evidenced by a moderate rise of blood pressure from 15 mm to 20 mm Hg). When the pressure remained steady at this level, *Rauwolfia* alkaloids were injected one by one and the pressure changes compared.

The result thus obtained confirmed our previous findings in the other two methods. Sinus paralysis could not, however, produce the same degree of rise of

the carotid pressure as after adrenalin or ephedrine and naturally the degree of hypotension produced by the *Rauwolfia* alkaloids was less marked

### DISCUSSION

Various effects of the *Rauwolfia* alkaloid have been studied in the present paper. An analysis of these actions shows that the plant possesses two groups of active principles of distinct pharmacological actions. The first group consists of ajmaline and serpentinine which raise the blood pressure, depress the cardiac musculature, produce splenic contraction, stimulate the respiratory movements and the peristalsis of the isolated guinea-pig intestine and intestine *in situ*. The second group which consists of serpentine produces an opposite effect on all the organs enumerated above. The total alkaloids as also the alcoholic extract of the plant behave like serpentine.

These varied actions naturally raise certain hypotheses as to the probable seats of action of these drugs. One could be tempted to attribute the mechanism of the vascular effects to the parasympathetic and sympathetic autonomous nervous systems, rise of blood pressure with ajmaline and serpentinine, and hypotension produced by serpentine. But when the intestinal effect is associated with the cardiovascular action and the effect of atropinization is taken into consideration, it becomes obvious that a perfect parasympatho-mimetic or lytic effect does not occur, the intestinal effect resembling a direct muscular action. Certain properties also suggest the possibility of a sympathetic and adrenergic effect, e.g. vasoconstriction (ajmaline and serpentinine) and partial reduction of the pressor effect after ergotoxine. But the stimulating action on the intestines, incomplete reversing of adrenalin action by an ajmaline, prejudice this conclusion. Under the circumstances it is not possible to identify adrenergic or cholinergic effect in these alkaloids and classify them as mimetic or lytic poisons of the parasympathetic or sympathetic nervous systems, though certain characteristics are present. Further elucidation is necessary before this issue can be finally settled.

Nevertheless, these alkaloids present definite indications of marked vasomotor actions, as evidenced by blood pressure and organ volume changes. Plethysmographic studies showed that splanchnic vasoconstriction occurred with ajmaline and serpentinine along with the rise of the blood pressure, and vasodilatation occurred when the pressure was depressed by serpentine, total alkaloids and the alcoholic extract. Elucidative explorations conducted for this issue with a view to decide between the central and peripheral effect by spinal and decerebrate preparations and effects of adrenalin and ergotoxine on these drugs, show that the hypertensive properties of ajmaline and serpentinine change to hypotension in spinal preparations but remain unaltered in decerebrate animals. Serpentine action remains unaltered in both these conditions. Ergotoxine definitely reverses the action of serpentine and ajmaline can partially reverse the action of adrenalin. These data suggest that a varying degree of both central and peripheral effect is likely to be present in these alkaloids.



As to the hypotensive effect of the *Rauwolfia* alkaloids a comparative evaluation of their relative properties has been possible in this work. We have found that both in normal cats as well as in cats with induced hypertension, serpentine proved to possess the maximum pressure-reducing properties and could accordingly be considered to be the hypotensive principle of the drug. Hypotensive action of the total alkaloids and of the alcoholic extract seemed to be due to the presence of serpentine in them as its removal along with that of ajmaline and serpentinine from the total alkaloids left the remaining portion entirely ineffective on the blood pressure. Attempts to eliminate serpentine from the total alkaloids are now being made by the Chemistry Department of this Institution, which will enable us to demonstrate more conclusively if this hypothesis is correct.

Our results, in this respect, do not fully agree with the earlier findings of Chopra and Chakravarti (*loc cit*) who, working on pithed and decerebrate cats, came to the conclusion that ajmaline was the major pressure-reducing factor in experimental hypertension. The reason why tests on normal cats should lead to a different conclusion is difficult to explain.

Analysis of the intestinal action of *Rauwolfia* alkaloids demonstrated that both ajmaline and serpentinine, which stimulated the intestine, could account for the laxative effect of the drug, so often observed by clinicians.

#### CONCLUSIONS

1 The different active constituents of *R. serpentina* have been studied and compared in this paper.

2 The extract, total alkaloids and serpentine show marked hypotensive properties, ajmaline and serpentinine, on the contrary, are hypertensors. In experimental hypertension all of them induced hypotension, serpentine producing the maximum effect amongst them.

3 Serpentine could be considered to be the chief pressure-reducing factor amongst *Rauwolfia* alkaloids.

4 Ajmaline and serpentinine stimulate, whereas serpentine depresses, the intestine. The first two would account for the purgative effect of the extract, which has often been reported in the Ayurvedic literature.

5 The various effects suggest that the *Rauwolfia* alkaloids probably act on the vasomotor system and also directly on plain muscles of the blood vessels and intestines.

#### ACKNOWLEDGMENT

Our thanks are due to Dr S. Siddiqui for the supply of alkaloids.

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# STUDIES ON SOME DEXTRO-ROTATORY HYDROCUPREIDINE DERIVATIVES

## Part III

### COMPARATIVE EFFECTS ON *PARAMÆCIA CAUDATUM*

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[Received for publication, November 25, 1941]

CHOPRA, MUKERJI and CHAKRAVARTY (1938) and Mukerji and Iyengar (1938) reported upon the comparative activity of some hydrocupreidine derivatives on red blood corpuscles and on digestive enzymes. The present paper reports a continuation of this study with a view to find out if these derivatives could be utilized for purposes (e g against protozoa and bacteria) for which hydrocupreine salts have been recommended by Morgenroth *et al* (1922-1926)

## EXPERIMENTAL METHODS

*Paramæcia culture*—Small pieces of hay thoroughly washed in tap-water, were placed in a conical flask and boiled for ten minutes with 300 c c of water. The mouth of the flask was covered with a clean gauze and the fluid on cooling was inoculated with a culture of *Paramacium caudatum*. After a week innumerable actively motile ciliated organisms were found in the solution.

*Preparation of solution*—Higher homologues of hydrocupreidines being sparingly soluble in water these were first dissolved in minimum quantity of

deci-normal hydrochloric acid and re-distilled water was then added to make up the required dilutions

*Hydrogen-ion concentration* — Acton (1921) demonstrated that the pH of a solution has considerable influence on the lethality on *P. caudatum*, an increase on the acid side being associated with a corresponding increase in killing power of the solution. The hydrogen-ion concentration of the solutions was therefore very carefully determined electrolytically by means of the quinhydrone electrodes. As it was not possible to bring the pH of all the solutions to the neutral point owing to their meagre solubility, attempts were made to bring the pH as near to the neutral point as practicable by careful addition of phosphate buffer. In this way, pH of the solutions was maintained between 5.3 and 6.4. Controls with N/10 HCl of the same pH were run side by side to avoid any fallacy in the results.

*Effects on paramœcia* — Equal quantities (2 drops of each) of paramœcia culture and the solution under test were mixed thoroughly on a moated glass-slide. A vaseline ring was made around the cover-slip to avoid early dehydration of the preparation. The lethal time, as indicated by the paramœcia becoming completely motionless, was noted by means of a stop-watch. The comparative lethality was recorded in terms of quinine hydrochloride. Corresponding hydrocupreine derivatives where available, have also been studied side by side.

## RESULTS

From Table I, it is evident that the hydrocupreidine derivatives have powerful toxic effects on undifferentiated protoplasm and the activity of individual members tends to increase as we go higher up in the series. The lethal effect on paramœcium is definitely influenced by the number of carbon atoms in the side chain and, in this respect, it runs parallel with the hæmolytic activity of these salts (Chopra *et al.*, *loc. cit.*). The behaviour of *iso*-amyl, normal heptyl and normal octyl derivatives appear to be somewhat peculiar, in that the *n*-heptyl and *n*-octyl derivatives are less toxic than their positions in the homologous series would indicate, whereas the *iso*-amyl salt is comparatively much more toxic. It is also seen, contrary to general conceptions, that the *iso*-compounds are less toxic than their normal derivatives. From Table II, it will be seen that the lævo-rotatory hydrocupreines are more toxic to the ciliates than their corresponding dextro-rotatory hydrocupreidines, but the intensity of action of the lævo-rotatory compounds in this group is by no means as pronounced as is observed, for example, in the sympathomimetic amine series (e.g. adrenalin) where the lævo-derivatives are often 10 times or more stronger than the corresponding dextro-derivatives.

The higher members of the hydrocupreidine series (from butyl upwards) which are not only powerful inhibitors to amylase but also possess powerful hæmolytic activity, cannot naturally be used for internal medication but whether their marked and intense cell-destroying power, which is generally very much stronger than quinine, can be conveniently utilized against micro-organisms as antiseptics or disinfectants remains to be worked out.

TABLE I

*Effect of dectro-rotatory hydrocupreidine derivatives on Paramoecium caudatum*

Name of compound	DILUTION EMPLOYED AND TIME TAKEN TO KILL PARAMOECIA									
	* 1/5,000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/200,000	1/400,000	1/800,000	
Quinine hydrochloride	20 hours	† No death	No death	No death	No death	No death	No death	No death	No death	No death
Hydrocupreidine	7 minutes	10 minutes	13 minutes	75 minutes	No death	No death	No death	No death	No death	No death
Methyl hydrocupreidino	5 minutes	7 minutes	9 minutes	33 minutes	57 minutes	No death	No death	No death	No death	No death
n propyl hydrocupreidino	2 minutes	2 minutes	3 minutes	5 minutes	15 minutes	30 minutes	43 minutes	79 minutes	Majority died in 5 hours	
Isopropyl hydrocupreidino	3 minutes	4 minutes	6 minutes	15 5 minutes	25 5 minutes	37 minutes	50 minutes	Majority died in 5 hours	No death	
n butyl hydrocupreidine	Instantaneous	2 minutes	2 5 minutes	3 minutes	4 minutes	13 minutes	15 minutes	50 minutes	No death	
Isobutyl hydrocupreidino	1 minute	1 minute	3 minutes	4 minutes	8 minutes	10 minutes	29 minutes	10 3 minutes	5 hours	

NB—l-thyl hydrocupreidine was not available at the time of experiment

\* The strength given is that of the resulting mixture which is half that of the original strength used

† 'No death' indicates paramoecium did not die within 5 hours unless specially mentioned

TABLE I—*concl'd*

Name of compound	DILUTION EMPLOYED AND TIME TAKEN TO KILL PARAMOEIA									
	* 1/5,000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/200,000	1/400,000	1/800,000	
n amyl hydrocupreidine	Instantaneous	2 minutes	2 minutes	3 minutes	8 minutes	13 5 minutes	24 minutes	65 minutes	† No death	
Isobutyl hydrocupreidine	Instantaneous	Instantaneous	1 minute	2 minutes	6 minutes	7 minutes	15 minutes	35 minutes	No death	
n-hexyl hydrocupreidine	Instantaneous	1 5 minutes	2 minutes	3 minutes	5 minutes	7 minutes	17 minutes	83 minutes	No death	
n heptyl hydrocupreidine	Instantaneous	3 5 minutes	7 minutes	12 minutes	16 minutes	36 minutes	No death	No death	No death	
n octyl hydrocupreidine	1 minute	9 minutes	19 minutes	28 minutes	31 minutes	38 minutes	94 minutes	No death	No death	
Secondary octyl hydrocupreidine	Instantaneous	30 seconds	3 5 minutes	6 minutes	12 minutes	20 minutes	24 minutes	131 minutes	No death	

NB—Ethyl hydrocupreidine was not available at the time of experiment

\* The strength given is that of the resulting mixture which is half that of the original strength used

† 'No death' indicates paramoecium did not die within 5 hours unless specially mentioned

TABLE II

*Effect of laevo rotatory hydrocupreine derivatives on Paramecium caudatum*

Name of compound	DILUTION EMPLOYED AND TIME TAKEN TO KILL PARAMECIA									
	* 1/5,000	1/10,000	1/20,000	1/40,000	1/80,000	1/160,000	1/200,000	1/400,000	1/800,000	
Hydroquinone	1 15 seconds	4 minutes	7 minutes	15 minutes	35 minutes	55 minutes	† No death	No death	No death	
Ethyl hydrocupreino	1 minute	3 minutes	7 minutes	14 minutes	25 minutes	30 minutes	35 minutes	No death	No death	
Isobutyl hydrocupreino	30 seconds	30 seconds	1 minute	3 5 minutes	6 minutes	30 minutes	No death	No death	No death	
Isopropyl hydrocupreino	Immediate	Immediate	1 minute	2 5 minutes	3 minutes	6 minutes	25 minutes	No death	No death	
Isopentyl hydrocupreino	Immediate	Immediate	5 minutes	8 minutes	13 minutes	20 minutes	No death	No death	No death	
Isooctyl hydrocupreino	7 5 minutes	29 minutes	No death	No death	No death	No death	No death	No death	No death	

\* The strength given is that of the resulting mixture which is half that of the original strength used

† 'No death' indicates paramaecium did not die in 5 hours unless specially mentioned

## SUMMARY AND CONCLUSIONS

1 Comparative toxic action of hydrocupreidine derivatives and their corresponding available lævo-rotatory hydrocupreines on undifferentiated protoplasm (e.g. *P. caudatum*) were studied and compared with quinine hydrochloride

2 Hydrocupreidine derivatives are powerful protoplasmic poisons and in *in vitro* experiments, these are found to possess lethal properties of a very high order compared to quinine

3 In general, the toxicity of hydrocupreidine derivatives on unicellular organisms increases with the increase of carbon atoms in the side chain. Iso-derivatives in this series appear to be less toxic than their normal homologues though the difference in the intensity of action is not often very well marked

4 Lævo-rotatory hydrocupreines are more toxic to the ciliates than their corresponding dextro-compounds

5 In view of the high dilutions in which these hydrocupreidine derivatives are potent against free-living ciliates, their therapeutic utilization as disinfectants in wound infections and surface antisepsis deserves further investigation

## ACKNOWLEDGMENT

This study was started at the request of Brevet-Colonel Sir Ram Nath Chopra, C I E, I M S (*Retd.*), and it is a pleasure to record our appreciation of the help and advice given by him at every stage

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## THE CHEMICAL INVESTIGATION OF THE GUM RESIN FROM *BALSAMODENDRON MUKUL* HOOK

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THE plant *Balsamodendron mukul* Hook belongs to the family Burseraceae and is synonymous with *Commiphora mukul* Engl (Wehmer, 1935). It is a small tree, about 4 feet to 6 feet high, with brown flowers. It grows in the arid zones of Rajputana, Khandesh, Sind, Kathiawar, East Bengal and Assam. The gum resin from the plant, known in Sanskrit as Koushikaha or Guggulu and in Bengali and Hindi as Guggul, is obtained by incision of the bark during the cold season. It is of a brown or dull-green colour and has a bitter taste with an aromatic odour. It is used as a demulcent, aperient, carminative and alterative, and it is stated to be useful in leprosy, rheumatism, syphilitic disorders, scrofulous affections, nervous and skin diseases and in urinary disorders. An ointment prepared from this is used for bad ulcers, especially in the treatment of Delhi sores (Watt, 1889-96).

This investigation was suggested by an inquiry from the Government of India to find out useful substitutes for drugs whose imports have been curtailed owing to the present war, and the present drug was taken up in order to find out whether it could be used as a substitute for Balsam of Tolu.

There seems to be no record of any detailed chemical examination of this gum resin and the constituents of the gum resin from an allied plant, *Commiphora myrrha* Holm (syn *Balsamodendron myrrha* Nees), are stated to be gums 40 to 60 per cent, resins 27 to 50 per cent, essential oil 2.5 to 10 per cent, bitter substance etc (Wehmer, *loc cit*). The constituents of the Balsam of Tolu have been found to be about 7.5 per cent of an oily aromatic liquid consisting mainly of benzyl



benzoate and a small proportion of benzyl cinnamate, about 3 per cent of impurities, about 0.05 per cent of vanillin, about 12 to 15 per cent of free cinnamic acid and benzoic acid, the latter being present in smaller amounts, the resin was present as an ester which on hydrolysis gave cinnamic acid and a little benzoic acid and a resin alcohol toluresinotannol  $C_{17}H_{18}O_5$  (Oberlander, 1894)

The material used for the present work was secured from the local market and the crude drug was freed mechanically from foreign matter, such as pieces of wood, sand etc., which amounted to about 4.65 per cent, before it was used for the investigations

#### EXPERIMENTAL

*Moisture and volatile matter* —When dried at  $105^{\circ}\text{C}$  it lost 7.72 per cent of its weight

*Ash* —On ignition it burnt with a smoky flame emitting an aromatic odour and left behind 19.49 per cent of ash. The ash was strongly alkaline, and on analysis was found to contain 59.0 per cent of insoluble siliceous matter, 21.26 per cent of lime calculated as  $\text{CaO}$ , 2.1 per cent of magnesia ( $\text{MgO}$ ), and 6.88 per cent of iron and alumina ( $\text{Fe}_2\text{O}_3$ ,  $\text{Al}_2\text{O}_3$ )

*Gum* —A weighed quantity of the substance was dissolved in water, filtered and precipitated with alcohol. The precipitate was allowed to settle and the clear supernatant liquid was decanted off. The precipitate was dissolved in water and reprecipitated with alcohol and the process was repeated for a third time. Finally, the precipitate was thoroughly washed with dilute alcohol, filtered, dried and weighed. The gum obtained amounted to 32.4 per cent.

*Essential oil* —One hundred and forty grammes of guggul were distilled in steam until the distillate became clear. The aqueous distillate was extracted with ether, the ether removed and the residue dried and weighed. The yield of the essential oil amounted to 1.45 per cent.

*Resin* —The substance was saponified with alcoholic potash, the product diluted with water, transferred to a separating funnel and extracted with ether. The ethereal solution was washed twice with water with two successive portions of dilute aqueous potash and finally with distilled water. The ether was removed and the residue dried at  $110^{\circ}\text{C}$  to a constant weight. The non-saponifiable resin thus obtained amounted to 15.8 per cent. The saponified product, after removal of the non-saponifiable portion, was transferred to a beaker and precipitated with  $\text{HCl}$ , only a very small quantity of a precipitate could be obtained.

*Solubility* —The results of the solubility of the gum resin in various solvents are shown in Table I and compared with those for Tolu Balsam, the figures for the latter being those given by Dieterich (1920) —

TABLE I

Solvent	PER CENT SOLUBLE	
	Guggul	Tolu Balsam
Alcohol (90 per cent)	24.89	Complete
Sulphuric ether	24.12	53 to 88 (Dieterich, 1920), complete (Oberlander, 1894)
Acetic ether	26.23	Complete
Petroleum ether	9.89	2.2 to 10.2
Chloroform	26.67	Almost complete
Benzene	24.07	82 per cent to almost complete
Carbon disulphide	18.72	19.6 to 35

**Acid value**—One gramme of the substance was taken in a glass stoppered flask and treated with 10 c c N/2 alcoholic potash and 10 c c of N/2 aqueous potash, 50 c c of benzene of specific gravity 0.700 was added and the mixture allowed to stand for 24 hours with occasional shaking. Five hundred c c of water was then added and titrated with N/2 sulphuric acid, using phenolphthalein as indicator. Alkali required for neutralization =  $0.7 \times 0.992 = 0.6944$  c c, hence the acid value = 9.74

**Saponification value**—Two grammes of the substance were boiled under reflux with 30 c c water and 25 c c N/2 alcoholic potash (factor 0.992). N/2 sulphuric acid required for back titration = 20.5 c c, hence the saponification value = 60.3

**Ester value**—The difference between the saponification value and the acid value ( $60.3 - 9.74$  or 50.56) gave the ester value

**Unsaponifiable matter**—2.5 grammes of the substance were heated with 25 c c N/2 alcoholic potash for one hour. The saponified product was diluted with 50 c c water and extracted thrice with ether. The ethereal extracts were washed with water, with N/2 aqueous potash and finally with water. The ether was removed and the residue carefully freed from solvent, dried and weighed. The unsaponifiable matter in the sample amounted to 16.99 per cent.

**Ethereal extract of the gum resin**—Five hundred grammes of guggul were extracted repeatedly with sulphuric ether until it was completely exhausted. The ether was removed and the residue, a yellow viscous liquid, was dried in a vacuum desiccator.

**Acid value of ethereal extract**—One gramme of extract was dissolved in 50 c c alcohol and titrated with N/2 potash using phenolphthalein as indicator. The acid value was found to be 4.20.

**Saponification value of ethereal extract**—Two grammes of extract were refluxed with 25 c c N/2 alcoholic potash and then titrated with N/2 sulphuric acid. The saponification value was found to be 64.1.

**Non saponifiable matter of ethereal extract**—The value found was 75.5 per cent.

**Ester value of ethereal extract**—The ester value calculated from the saponification and acid values was found to be 59.9.

**Optical activity of ethereal extract**—A 5 per cent solution of the extract in absolute alcohol gave  $[\alpha]_D^{25} = +26.3^\circ$ .

**Examination of the gum resin for its constituents**—Two kilograms of the substance were extracted thrice with ether. The ethereal extracts combined filtered from suspended impurities and washed repeatedly with a 0.25 per cent solution of caustic soda. The ethereal solution was then washed with water and extracted twice with a saturated solution of sodium bisulphite. The bisulphite solution was decomposed with cold dilute sulphuric acid (3 vols of acid to 5 vols of water). When the evolution of  $\text{SO}_2$  ceased it was heated on the water bath to remove the last traces of  $\text{SO}_2$ . After cooling, the solution was extracted with sulphuric ether. The ethereal solution washed with water and the ether removed at a low temperature. There was no appreciable amount of residue indicating the absence of any aldehyde.

The ethereal extract after treatment with aqueous sodium bisulphite was washed with water until neutral, dehydrated with sodium sulphate and the ether removed. A dark brown, thick, oily liquid was obtained as residue.

Twenty grammes of the above oily liquid were subjected to fractional distillation *in vacuo*. It began to distil at  $175^\circ\text{C}$ . The first fraction collected between  $175^\circ\text{C}$  and  $245^\circ\text{C}$  consisted of only a few drops of a yellow liquid. The second fraction distilling up to  $275^\circ\text{C}$  also consisted of a few drops of a deep yellow liquid. The third fraction collected up to  $290^\circ\text{C}$  consisted of about 3 c c of a dark brown liquid. Above  $290^\circ\text{C}$  the liquid began to decompose. A further quantity of fresh liquid was distilled in vacuum and the distillate up to  $290^\circ\text{C}$  was collected. All the fractions were mixed together and saponified with a 10 per cent solution of caustic potash. The saponified product was diluted with water and extracted with ether. The ethereal solution was washed with water and the ether removed. No appreciable amount of residue was obtained. The alkaline solution was acidified with sulphuric acid and a small amount of a precipitate was obtained. The precipitate was washed, dissolved in alcohol and allowed to crystallize slowly, but no crystals could be obtained. No evidence could be obtained about the presence of benzyl alcohol, cinnamic acid or benzoic acid in the saponified product. The alkaline solution obtained by shaking the ethereal solution of the original drug with 0.25 per cent caustic soda as mentioned before was freed from dissolved ether by warming on the water bath, cooled and a current of  $\text{CO}_2$  gas passed. A very small quantity of a yellow precipitate was obtained. This yellow resinous powder softened at  $93^\circ\text{C}$  and melted completely at  $121^\circ\text{C}$ . The alkaline solution was concentrated to a small bulk and allowed to stand for some days. A small quantity of a crystalline substance separated out. It was dissolved in alcohol decolorized with animal charcoal and allowed to crystallize. The crystals when purified by recrystallization from boiling water were obtained as colourless needles mp  $116^\circ\text{C}$  to  $118^\circ\text{C}$ . The quantity was too small for further work. The mother liquor from these crystals was made distinctly acid with  $\text{HCl}$  but nothing separated out.

## SUMMARY AND CONCLUSIONS

TABLE II

*Comparative table*

	Tolu Balsam	<i>Balsamodendron mukul</i> (guggul)	Ethereal extract of guggul
Free acid, per cent	7.1 to 16.3	Traces	—
Combined acid, per cent	19.4 to 37.7	—	—
Acid value, per cent	111.3 to 126	9.71	4.20
Ester value, per cent	70.0 to 88.1	50.66	59.9
Saponification value per cent	188.6 to 204.3	60.3	64.1
Saponification value of ethereal extract, per cent	247.4 to 301.8	—	—
Gum, per cent	—	32.4	—
Specific rotation	—	—	+ 26.3°
Essential oil, per cent	0.2	1.45	—
Ash, per cent	—	19.49	—

The sample of *Balsamodendron mukul* was found to contain about 4.65 per cent of foreign impurities, 32.4 per cent of gum, 19.5 per cent of mineral matter consisting chiefly of  $\text{SiO}_2$ , Ca, Mg, Fe and Al. It contains about 1.45 per cent of an essential oil having a faintly aromatic odour. The presence of benzoic acid, cinnamic acid, benzyl benzoate, benzyl cinnamate or vanillin, which form important constituents of Tolu Balsam, could not be detected in *Balsamodendron mukul*. Besides a very small amount of a saponifiable resin, the balsam contains resins, which could not be saponified. The results indicate that *Balsamodendron mukul*, which does not contain the constituents which are stated to be the physiologically active components of the Balsam of Tolu, cannot be recommended as a substitute for the latter.

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 Supplement

## ESTIMATION OF AMMONIA NITROGEN IN BIOLOGICAL MATERIALS

BY

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[Received for publication, October 13, 1941]

DURING studies on the autolysis of different glands and tissues, an attempt to determine ammonia did not give satisfactory results when estimated by the usual magnesia-distillation method. Known amounts of ammonia were then added to various protein materials, such as freshly slaughtered beef, veal etc (thus giving them minimum chance to decompose), but the recovery of the added ammonia always gave higher figures than the actual amount added when estimated by the magnesia method. It appeared that probably the conditions under which such estimations were carried out were drastic and the highly alkaline reaction (pH 10 to 11) which was produced by the use of magnesia was conducive to the hydrolysis of protein. Accompanying this hydrolysis ammonia was evolved and this gave higher results than the actual amount initially present in the sample under examination.

Since the high alkalinity of magnesia might play a part in the hydrolysis of protein, it was thought that if the pH of the distilling solution could be brought and maintained slightly on the alkaline side, the decomposition of the nitrogenous bodies would be minimized. It was evident that as the concentration of the reagents proceeded with distillation, pH would go higher and higher if the reaction was adjusted with a single base even just on the alkaline pH. The other alternative was to use a buffered solution for the adjustment of the reaction. Nichols and Foote (1931) and Shrikhande (1941), while working with trade waste, decomposed plant materials etc, have reported that ammonia determinations were unsatisfactory if carried out in presence of magnesia or sodium carbonate because of the high alkalinity and suggested the use of a phosphate buffer of pH 7.4 for ammonia estimation. An investigation to determine ammonia nitrogen in presence of protein with phosphate buffer was accordingly undertaken and the results are given herein.

## EXPERIMENTAL

To see which reaction was most suitable for the accurate recovery of ammonia in presence of protein, 20 mg of ammonia in the form of ammonium sulphate were added to 10 c c of horse serum and the distillations were carried out at different pH adjusted by phosphate buffer solution. The results are given in Table I —

TABLE I

pH	RECOVERED AMMONIA IN MILLIGRAMS	
	With horse serum	Without horse serum
7.0	19.4	19.2
7.4	20.1	20.0
8.0	21.6	20.0
8.5	23.2	20.0

It was thus evident that pH 7.4 was the most suitable reaction and this pH was used in all subsequent estimations.

*Preparation of phosphate buffer solution of pH 7.4*

14.3 g of potassium di-hydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and 91.0 g of di-potassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ ) were dissolved in ammonia-free distilled water and the volume made up to one litre.

The following materials were taken for experimental work, each representing a typical class —

- (a) Autolysed beef
- (b) Horse serum
- (c) Culture filtrate—*Cl. tetani* toxin
- (d) Bacto-peptone

Each material was taken in a round-bottom flask, 150 c c buffer solution were added and the volume made up to about 350 c c with ammonia-free distilled water. In each case, prior to distillation, 20 mg of ammonia (in the form of ammonium sulphate) were added as control and the distillation was finished in the usual way. A blank was also made in each case without the addition of ammonia. Another set of experiments were carried out with magnesia (3 g) exactly in the same way.

as in the case of phosphate buffer solution Results of ammonia recovery are given in Table II —

TABLE II

Materials	Blank $\text{NH}_3$ in mg	Addition of $\text{NH}_3$ in mg	AMMONIA RECOVERED IN MG	
			Phosphate buffer	Magnesia
(1) Autolysed beef (10 g)	2.64	20.0	22.78	24.22
(2) Horse serum (50 c.c.)	Nil	20.0	20.10	31.16
(3) Toxin ( <i>Cl. tetani</i> ) (20 c.c.)	18.10	20.0	38.10	38.50
(4) Bacto peptone (10 g)	6.12	20.0	26.06	27.68

Pure protein was then taken to see whether the increased amount of ammonia was also obtained in this case with magnesia. Pure serum globulin was prepared by precipitating horse serum (50 c.c.) with 23 per cent sodium sulphate solution. The precipitate was washed with sodium sulphate solution till the wash-water gave no biuret reaction. The precipitate was dissolved in water and equal volumes were taken for ammonia estimation, both with buffer solution and with magnesia. Results are recorded in Table III. In each case 20 mg. of ammonia were added as control —

TABLE III

	Ammonia added in mg	Ammonia recovered in mg
Magnesia	20.0	23.4
Phosphate buffer	20.0	20.4

Different volumes of phosphate buffer were then used as distilling reagent to ascertain whether there was any variation in ammonia figure with the increase in

the amount of buffer solution Twenty mg of ammonia as ammonium sulphate were added in each case as usual Results are recorded in Table IV —

TABLE IV

Materials	NH <sub>3</sub> added in mg	NH <sub>3</sub> RECOVERED IN MG WITH BUFFER SOLUTION	
		200 c c	250 c c
Autolysed beef (10 g)	20 0	22 78	22 78
Horse serum (50 c c)	20 0	20 20	20 20
Toxin ( <i>Cl tetani</i> ) (20 c c)	20 0	38 10	38 10
Bacto peptone (10 g)	20 0	26 06	26 06

## DISCUSSION

These results leave no doubt that the high alkalinity of magnesia causes an apparently high yield of ammonia due to the hydrolytic de-amination of protein and its break-down products This factor can be practically eliminated by the use of a phosphate buffer of pH 7.4 as distilling reagent Moreover, no variation in the amount of ammonia recovered was noticed with different volumes of the buffer solution (*vide* Table IV)

It is interesting to note from Table II, that marked variations in the yield of ammonia were noticed in cases of serum and meat (containing crude protein) than in cases where protein is in the form of break-down products (peptones etc) In case of *Cl tetani* toxin, practically no variation in the amount of ammonia recovered was noticed with buffer solution and with magnesia It was possibly due to the small amount of nitrogenous bodies present in it and that also in the form of break-down products (Basu and Sen, 1941)

It was argued that higher figure for ammonia might be due to some factor other than the high alkalinity of magnesia, but results recorded in Table III, with pure globulin, definitely showed that the de-amination of protein was mainly due to the high alkaline reaction of magnesia

## SUMMARY

From the results so far recorded it is evident that the magnesia-distillation method is not suitable for the estimation of ammonia in the presence of protein The distillation, if carried out in the presence of phosphate buffer of pH 7.4, gives more accurate results

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## A MODIFIED METHOD FOR THE DETERMINATION OF 'PROTHROMBIN TIME'

BY

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[Received for publication, November 25 1941]

SINCE the isolation of vitamin K and its therapeutic utilization in certain hæmorrhagic conditions, the determination of the prothrombin level in blood has attracted considerable attention. Quick (1938) described a method for estimating the prothrombin time which can be briefly represented as follows —

$$(1) \frac{0.1 \text{ c.c. plasma (Oxalated)}}{\text{Oxalated}} + \frac{0.1 \text{ c.c. thromboplastin sol. (Prepared from rabbit's brain)}}{\text{Prepared from rabbit's brain}} + 0.1 \text{ c.c. } \frac{M}{40} \text{ CaCl}_2 \text{ —————}$$

Appearance of clot (prothrombin time) = 12 to 13 seconds

The results obtained by this method have often been found to be rather divergent. Attempts have therefore been made to modify the method so as to secure more uniform and dependable results. The chief disadvantages in the Quick's method are (1) The solution is rather opaque on account of the use of the rabbit brain extract as the source of thromboplastin. The end-point in this test is the formation of the fibrin web. This end-point cannot be accurately observed in such an opaque solution. (2) A fresh thromboplastin preparation has to be obtained every time a determination is made. This is not only inconvenient and time-consuming, but is also open to the objection that the thromboplastin preparation employed in the reaction being freshly made each time may and actually does vary in potency from batch to batch. In a standard technique on which the interpretation of



data of diagnostic and prognostic value will depend, such a variation in potency of a reaction component is obviously not desirable

Fullerton (1940) has recommended a method wherein he uses Russell's viper venom as the thromboplastin instead of rabbit brain extract. The method can be briefly represented as follows —

$$(B) \frac{0.2 \text{ c.c. plasma}}{(\text{Oxalated})} + \frac{0.2 \text{ c.c. Russell's viper venom}}{(1 \text{ in } 10,000)} + 0.2 \text{ c.c. } 0.025 \text{ M CaCl}_2 \text{ —————}$$

Appearance of clot (prothrombin time) = 18 to 25 seconds

In the above method it is considered necessary by the author to use a freshly prepared solution every time a prothrombin test is performed, as otherwise the solution is liable to rapid deterioration in thromboplastic potency. A fresh ampoule containing viper venom (0.1 mg) in dry form (commercially available as 'Stypven' or 'Russven', B. W. & Co. or Boots Pure Drug Co.) should be dissolved in 1 c.c. distilled water immediately before use. The chief disadvantage of this method is that the prothrombin time is often delayed. Hobson and Witts (1940) report that when Russell's viper venom alone is used the range of variation is greatly increased and the results in hæmorrhagic diathesis may be grossly misleading. Hence they have suggested the addition of lecithin to the venom solution. Their method can be represented as follows —

$$(C) 0.1 \text{ c.c. plasma} + 0.1 \text{ c.c. Russell's viper venom (1 in 20,000) lecithin} \\ (5 \text{ mg /c.c. venom}) \text{ reagent} + 0.1 \text{ c.c. } 0.025 \text{ M CaCl}_2 \text{ —————}$$

Appearance of clot (prothrombin time) = 8 to 11 seconds

The reason for the variation of results in Fullerton's method might be due to the fact that the solid venom in each ampoule may have been derived from different batches which might vary in their relative thromboplastic potencies. The second disadvantage in Quick's method is thus not eliminated altogether in Fullerton's method, although it can be claimed that the thromboplastin is much more uniform. If it is possible to use a stock solution of venom whose thromboplastic potency has once been standardized by a series of prothrombin determinations, Fullerton's method can be regarded as of definite advantage. It is stated by Fullerton that the efficacy of the venom deteriorates rapidly in solution. No data showing any such deterioration are presented. The possibility or otherwise of using a standard stock solution of venom had therefore to be established.

While working on the relationship between plasma trypsin and blood coagulation, it was necessary to determine the prothrombin time in a number of physiological and pathological conditions. After many trials with both the Quick and the Fullerton techniques, it was realized that there is considerable room for the improvement of this useful clinical test. Experience with the Fullerton technique indicated that the method would yield quite satisfactory results, if the dilution of the prothrombin in the plasma could be reduced and the speed of the thromboplastin-prothrombin-calcium reaction accelerated. Both these objects will be served by directly adding to plasma 0.2 c.c. of 1 in 20,000 venom solution in 0.025 M CaCl<sub>2</sub> solution.

## EXPERIMENTAL

The stability of stock solutions of 1 in 20,000 Russell's viper venom in water or in 0.025 M  $\text{CaCl}_2$ , kept under toluene at a temperature of about  $5^\circ\text{C}$ , was studied in the first instance

*Stock solutions —*

(i) 0.1 M sodium oxalate solution

(ii) 0.025 M  $\text{CaCl}_2$  solution

\*(iii) 1 in 20,000 Russell's viper venom solution in water

\*(iv) 1 in 20,000 Russell's viper venom dissolved in 0.025 M  $\text{CaCl}_2$  solution

4.5 c.c. of blood fresh from a vein are pipetted into a centrifuge tube containing 0.5 c.c. of 0.1 M sodium oxalate solution and centrifuged after thorough mixing. The plasma is used for the determination of prothrombin.

0.2 c.c. of this plasma was added to 0.2 c.c. of 1 in 20,000 Russell's viper venom solution in water and 0.2 c.c. of 0.025 M  $\text{CaCl}_2$  solution and kept at a temperature of  $37^\circ\text{C}$  to  $38^\circ\text{C}$ . The interval in seconds between the addition of calcium solution to the mixture of plasma and venom solution and the earliest appearance of the fibrin web is taken as the prothrombin time. The stock solutions (iii) and (iv) were kept under toluene in a refrigerator at about  $5^\circ\text{C}$  and periodically taken out for use in the following experiments in order to test the stability of thromboplastic potency of the solutions. The prothrombin time was also simultaneously determined by our modified method, i.e.

0.2 c.c. plasma + 0.2 c.c. of stock solution (iv) —————

—————> Appearance of clot (prothrombin time)

TABLE I

*Showing stability of the thromboplastic potency of venom*

Date of test	Prothrombin time of human plasma using solution (iii)	Prothrombin time using solution (iv)
	Seconds	Seconds
14-1-41	11-12	9-11
18-1-41	13	10
22-1-41	12	10

\* Fresh Russell's viper venom obtained from Drug House (India) Ltd. and not 'Styprven' or 'Russven' was used. Judged from the lower prothrombin time obtained, this venom is apparently more potent than the other two brands of venom.

TABLE I—*concl'd*

Date of test	Prothrombin time of human plasma using solution (iii)	Prothrombin time using solution (iv)
	Seconds	Seconds
27-1-41	13	11
4-2-41	12-13	10
15-2-41	11	9
25-2-41	12	10
11-3-41	13	11
28-3-41	14	11

The above results definitely show that the thromboplastic potency of stock solutions of Russell's viper venom (1 in 20,000) either in water or in 0.025 M  $\text{CaCl}_2$  solution remains unaltered if kept under toluene at a temperature of about  $5^\circ\text{C}$

Having thus established the stability of dilute stock solutions of Russell's viper venom the use of a standardized stock solution of Russell's viper venom for routine determinations of prothrombin could be safely recommended. In order to evaluate the normal prothrombin time of human plasma, by the modified method, routine determinations in about 25 cases were carried out. For purposes of comparison, the prothrombin time was determined by the Fullerton technique also with the only difference that the stock solution of Russell's viper venom was employed.

TABLE II

*Showing comparative prothrombin time of normal human plasma by the two methods*

(1) 0.2 cc plasma + 0.2 cc 1 in 20,000 venom in 0.025 M $\text{CaCl}_2$	Prothrombin time in seconds	9	9	8	9	8	9	10	9	10	9	11	10	9	10	9
		10	9	8	10	18	12	19	10	11	9					
(2) 0.2 cc plasma + 0.2 cc 1 in 20,000 venom + 0.2 cc 0.025 M $\text{CaCl}_2$	Prothrombin time in seconds	11	11	10	10	11	11	12	12	13	12	14	12	11	12	11
		13	12	11	13	23	15	22	13	14	12					

The average prothrombin time by the modified method is about 9 seconds, while by the Fullerton technique the average comes to about 11 to 12 seconds. In every case the clotting time has been speeded up by the simultaneous addition

of thromboplastin and calcium The total volume of the reaction mixture in this case is reduced to 0.4 c.c. instead of 0.6 c.c. when the thromboplastin and the calcium solutions are added separately, thereby resulting in an increase in concentration of prothrombin in the mixture by about 33 per cent

#### ADVANTAGES OF THE METHOD

(a) It permits the employment of a stable stock solution of thromboplastin of constant potency In routine testing of a large number of samples, the availability of a ready-made standardized thromboplastin solution is often a real advantage

(b) The addition of thromboplastin and calcium in one solution abolishes the time interval of the thromboplastin-prothrombin reaction, which can take place only in the presence of calcium

(c) The 'prothrombin time' is speeded up on account of the increased concentration of prothrombin in the reaction mixture

(d) The clot formed is well marked, as the fibrinogen is not diluted to the same extent as in Fullerton's method

#### SUMMARY

1 The stability in thromboplastic potency of dilute solutions of Russell's viper venom, both in water and in 0.025 M  $\text{CaCl}_2$ , has been studied in detail The results obtained indicate that the solutions when kept under toluene at a temperature of about  $5^\circ\text{C}$  are quite stable and hence standardized stock solutions can be used for routine prothrombin determinations

2 A slightly modified method for the determination of prothrombin has been suggested The prothrombin time is slightly speeded up by the revised method

3 The advantages of the modification are enumerated

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## OBSERVATIONS ON FILARIASIS IN LAKHIPUR AND BINAKANDY TEA-GARDENS (CACHAR DISTRICT, LOWER ASSAM)

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[Received for publication, November 17 1941]

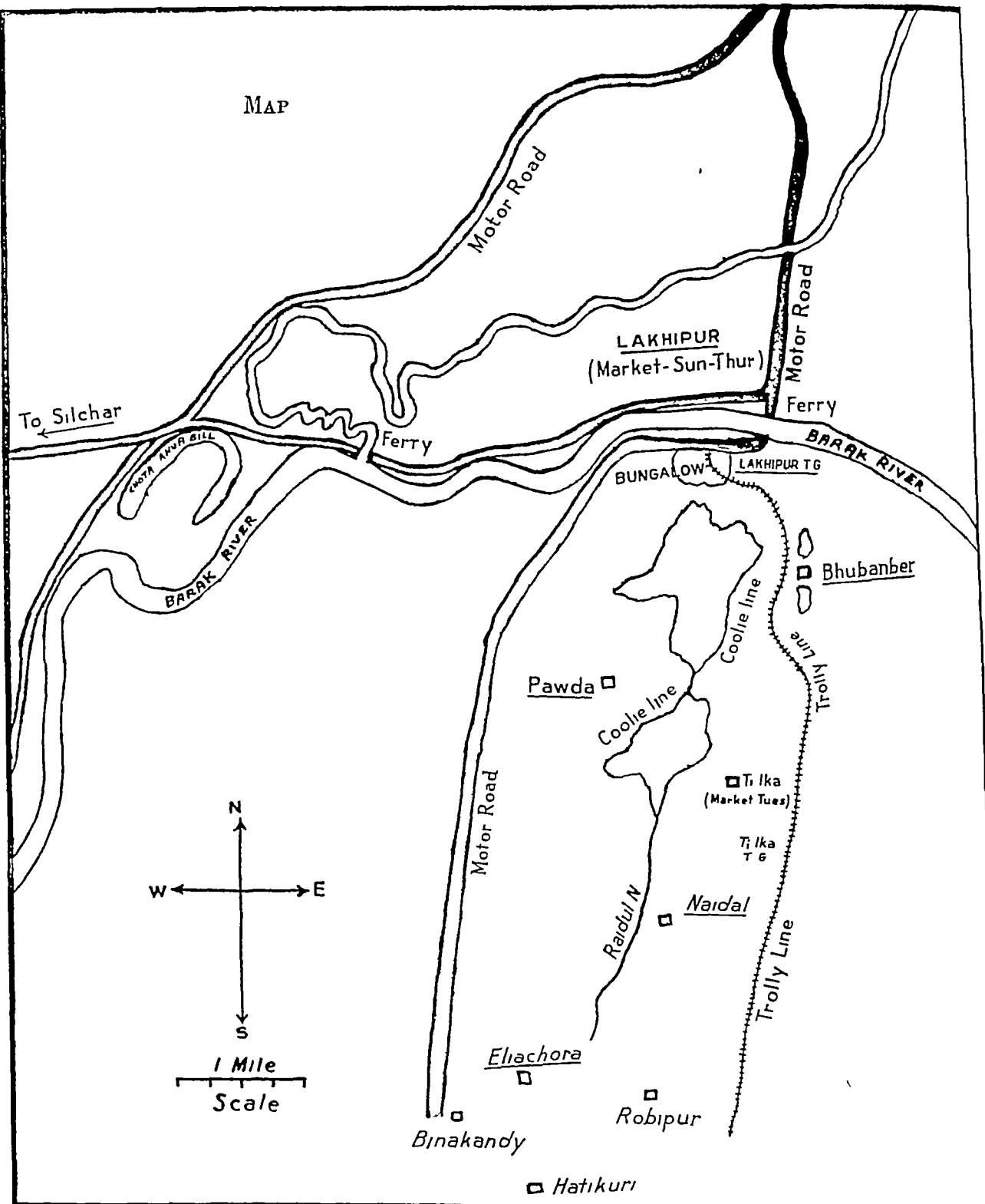
THE tea-gardens of Lakhipur and Binakandy are situated in the south-east of Cachar district in Lower Assam and comprise nine villages. In seven of these villages filariasis is endemic. The infection is chiefly *Wuchereria malayi*. It is said that these villages were quite free from filarial infection till about 30 years ago and the infection was introduced by imported labour from Bihar province (Fraser, 1938).

Buckley (Fraser, *loc cit*) observed the infection in these villages as due solely to *Mf malayi* in 1934, and Fraser, who made a thorough study of the infection and disease prevalent in these villages in 1936 and 1938, apprehended that the disease was on the increase. It was with a view to observing the rate of spread of the infection and also to see whether the infection had extended to the neighbouring villages that a survey of these villages was undertaken by the writer in February and March 1941.

The districts of Cachar in the east and Sylhet in the west form the plains of Surma valley of Lower Assam. The Cachar district lies between  $24^{\circ} 12'$  and  $25^{\circ} 50'$  North, and  $92^{\circ} 26'$  and  $93^{\circ} 29'$  East, covering an area of 3,767 square miles. The elevation of the land ranges from 70 to 300 feet above sea-level. The chief river is the Barak or Surma. The average rainfall is 130 inches per year. Minor floods with occasional major floods are common. The hottest months are June to September with an average mean temperature of  $83^{\circ}\text{F}$ , the coldest month is January, with a mean temperature of  $65^{\circ}\text{F}$ . Humidity ranges from 70 to 100 per cent for seven months in the year, April to October. The soil is fertile being for the most part clay and sandy. Rice and tea form the important products of cultivation (Ramsay, 1924).

The area that has been surveyed is shown on the Map. The villages surveyed were (1) Lakhipur, (2) Bhubanber, (3) Tilka, (4) Naidal, (5) Pawda, (6) Robipur,

# MAP



## POPULATION ACCORDING TO THE LATEST CENSUS (MARCH 1941)

	Male	Female	TOTAL		Male	Female	TOTAL
1 Lakhipur	401	394	795	6 Robipur	36	24	1,443
2 Bhubanber	143	113	256	7 Binakandy	789	674	901
3 Tilka	185	143	328	8 Hatikuri	506	445	341
4 Naidal	71	69	140	9 Eliachora	176	165	2,785
5 Pawda	294	238	532				
<b>TOTAL</b>	<b>1,094</b>	<b>957</b>	<b>2,051</b>	<b>TOTAL</b>	<b>1,487</b>	<b>1,308</b>	<b>4,816</b>
				<b>GRAND TOTAL</b>	<b>2,581</b>	<b>2,265</b>	

(7) Binakandy, (8) Hatikuri and (9) Elhachora These villages lie close to one another at distances not exceeding 1 to 3 miles and cover an area of about 28 square miles The total population of these villages is 4,846 according to the latest census (March 1941) The population consists of Bengali settlers and coolies recruited from all over India and comprises different classes of people, chiefly Bhakta, Bowri, Gond, Gowala, Kól, Bhil, Tel, Dom and Chamár, all of them Hindus in religion

Water-supply is obtained from wells and the rivers Barak and Raidul There are no drains nor conservancy and the people use scrub jungle or open ground for defæcation The prevailing diseases in the order of frequency are malaria, bowel diseases, worm infections, venereal diseases and leprosy

To determine the incidence of filarial infection in different villages, the people were examined at night between the hours of 9 p m and midnight and thick smears of peripheral blood from the finger were taken for subsequent examination A total of 2,445 persons, representing 50 per cent of the population, was examined both for signs of filarial disease and for blood infection The results have been tabulated (Table I) showing the details in regard to the microfilaria rate and the filarial disease rate for these nine villages —

TABLE I

*The filarial disease rate and microfilaria rate per village*

Village	Total number of persons examined	Number of persons with filarial disease	Filarial disease rate, per cent	Number showing micro filariæ	Number without filarial disease	Number showing micro filariæ	Microfilaria rate, per cent
Lakhipur	646	0	0	0	646	6	0.93
Bhubanber	208	9	4.3	0	199	14	6.73
Tilka	328	23	7.0	0	305	18	5.40
Naidal	131	29	22.14	6	102	31	28.24
Pawda	422	12	2.84	1	410	16	4.03
Robipur	53	3	5.66	1	50	9	18.87
Binakandy	191	0	0	0	191	0	0
Hatikuri	219	1	0.46	0	218	0	0
Elhachora	247	33	13.36	2	214	11	5.26
TOTAL	2,445	110	4.49	10	2,335	105	4.30

Out of 2,445 persons examined 115 had microfilaria in the peripheral blood at night, showing a gross infection rate of 4.7 per cent Of these 115 carriers five were *Wuchereria bancrofti* infection and the rest were *W. malayi* The former infection was found in only one village, viz Lakhipur In Lakhipur, out of six positives, five were *bancrofti* and one was *malayi*

Two villages, Naidal and Robipur, showed high infection rates of 28.2 and 18.9 per cent respectively The four villages Pawda, Elhachora, Tilka and Bhubanber, showed infection rates ranging from 4 to 7 per cent Lakhipur has



a very low infection rate (0.9 per cent), while filarial infection was absent in Binakandy and Hatikuri. The infections were due to *Mf malayi* with the solitary exception of Lakhipur where they were mostly *Mf bancrofti*. As regards the distribution of the infection in the sexes, no marked difference was noticed in the case of either.

Out of 2,445 persons examined for filarial diseases 110 (4.5 per cent) showed clinical signs of the disease. The types of filarial disease observed are shown in Table II -

TABLE II

*The types of filarial diseases found*

Elephantiasis of leg	Elephantiasis of arm	Elephantiasis of legs and arms	Hydrocele
89	16	4	1

The extreme paucity of genital affections is remarkable. Only one case of hydrocele out of a total of 110 cases of filarial diseases was observed. The patient came from Muzaffarpur (Bihar) which he visited almost every year. Neither the peripheral blood nor the hydrocele fluid showed microfilariae. The other cases were of elephantiasis and they gave a history of lymphangitis which recurred in a mild or severe form at varying intervals of time. Abscesses along the lymphatics were common and scars of abscesses were present in a great majority of these patients. There was no case of chyluria in the whole population at any time.

Enlarged glands in the inguinal epitrochlear or in both regions were present in the majority of the persons examined.

With a view to determining the variation of the incidence of filarial infection in these villages the findings of the present survey were compared with the figures of the previous years (Fraser, *loc cit*) in Table III -

TABLE III

*The microfilaria rates of the different villages*

Year	VILLAGE						
	Nardal	Robipur	Tilka	Bhubanber	Pawda	Lakhipur	Elachora
1934							11.8
1938	30.4	17.8	7.1	7.1	5.3		
1941	28.2	18.9	5.5	6.7	4.0	0.9	5.3

The above figures do not show any increase in the incidence of the infection in these villages. On the other hand, a comparison of the disease rates of the different villages shows that there is an increase in the number of cases of filarial diseases in these villages in 1941 (Table IV). The infection does not appear to have extended to neighbouring villages so far —

TABLE IV

*The number of cases of filarial diseases (elephantiasis only)  
in the different villages*

Year	VILLAGE				
	Naidal	Robipur	Tilka	Bhubanber	Pawda
1938	9	2	9	4	3
1941	29	3	23	9	12

In Ehachora there were 16 cases of elephantiasis in 1934 in a population of 396, while 33 cases of elephantiasis were found out of 247 persons examined in 1941.

#### MOSQUITO SURVEY

The following species of mosquitoes were found in these villages during the period. The specimens included wild mosquitoes caught from houses in the villages during the collection of blood smears, and those bred out from larvæ and pupæ collected from ponds and other water collections in the area. They were kindly identified by Dr M O T Iyengar D Sc (Madras) Entomologist, Public Health Department, Bengal, and by Dr I M Puri, Ph D (Cantab), Entomologist, Malaria Institute of India, Delhi.

They are *Mansonioides annulifera*, *M. uniformis*, *M. indiana*, *Culex fatigans*, *C. vishnu*, *C. tritaeniorhynchus*, *C. gelidus*, *C. sp.* *Ædimorphus verans*, *Armegeres obturbans*, *Banksinella lineatopennis*, *Anopheles hyrcanus* *A. subpictus*, *A. pallidus*, *A. hyrcanus* var *ingerrimus*.

The mansonioides species were found to breed on Pistia plants (*Pistia stratiotes* Linn.) and on 'Dol' grass (*Sacciolepis interrupta* Stapf Syn *Panicum interruptum* Willd.) in these villages.

It is interesting to note that *Culex fatigans* was found only in one village, Lakhipur, and in no other area in these gardens.

One remarkable fact observed during the survey was that villages situated in the midst of extensively cultivated land, such for example as Lakhipur, Binakandy and Hatikuri, were practically free from filarial infection, on the other hand, villages situated in the midst of swampy areas, e.g. Naidal, Tilka, Robipur and Ehachora, showed a high incidence of filarial infection.

## SUMMARY

A filarial survey of nine villages in the tea-gardens of Lakhipur and Binakandy in the south-east of Cachar district was carried out. The infection was due mainly to *Wuchereria malayi* infection. The lesions were characteristic of *malayi* infection, elephantiasis of the extremities being the only type of disease present in the area. There was no case of affection of the genitals nor of chyluria. In one village, viz. Lakhipur, there were cases of both *bancrofti* and *malayi* infection. As regards the distribution of the infection in the sexes, no marked difference was noticed in the case of either of these infections.

The infection rate, when compared with the figures for the previous years, was seen to remain steady, while it was observed that there was an increase in the number of cases of filarial diseases over previous years, in all the endemic villages. The infection does not appear to have spread to the neighbouring villages so far.

One remarkable fact observed during the survey was that villages situated in the midst of extensively cultivated land were practically free from filarial infection, while villages situated in the midst of swampy areas showed a high incidence of filarial infection.

## ACKNOWLEDGMENTS

The writer is indebted to Dr G. Fraser, M.D., Ch.B. (Edin.), D.T.M. & H., Chief Medical Officer, Lakhipur and Binakandy tea-gardens, and to his staff for the facilities and help given during the survey.

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## STUDIES IN GASTRIC ACIDITY IN INDIANS WITH ALCOHOL TEST-MEAL

BY

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[Received for publication, December 29, 1941 ]

THE study of gastric acidity is of great importance in the diagnosis of a variety of pathological states, and is being increasingly employed in clinical practice, in cases not only involving the gastro-intestinal tract, but also in all cases of anæmia. The alcohol test-meal has largely replaced the old gruel meal for this purpose. Records of normal gastric response to the alcohol test-meal in Indians are comparatively few and hence the present investigation was undertaken to study the gastric acidity in the inhabitants of the United Provinces in health and some pathological conditions.

### PREVIOUS WORK IN INDIA

The work done in India on Indians is briefly reviewed.

McCay in about 1918 was the first one to do some work on the gastric acidity of Bengalis. He found that an average rice-eating Bengali showed a low gastric acidity. Bhatt (1930) attempted to establish an Indian standard curve of acidity and found no significant difference in the acid-secretory response of Indians as compared with people in the west. Bhattacharyya (1933) confirmed McCay's

findings of a low acidity in Bengalis Gupta (1933) studied the general level of gastric acidity in various types of anæmias Dharmendra and Napier (1935) and Napier and Dharmendra (1936), working on gastric acidity in Indian asthmatics, found that the acidity was higher than the normal of the textbooks Napier and Das Gupta (1935), working on normal Indians in Calcutta and tea-garden coolies in Assam found that the mean acidity level was higher in Indians than in normal Europeans Rao (1937) carried out gastric analysis on 100 normal Indian males in South India and found a high level of acidity Test-meals used by the above-mentioned workers were all oatmeal gruel

Napier, Chaudhuri and Rai Chaudhuri (1938) studied the gastric acidity by using alcohol as test-meal in health and disease in India

### EXPERIMENTAL STUDIES

*The subjects*—The total number of subjects investigated in the present series was 121 They belonged almost exclusively to the two major communities There were 19 women to 102 men a proportion in which the two sexes usually seek hospital aid The subjects ranged from youngest of 18 years to oldest of 70 years

Seventy five cases out of 121 have been taken to represent normal Care was taken that none of these patients had any sign or symptom pertaining to the gastro intestinal tract, nor were they anæmic They had been admitted into the hospital with complaints unconnected with the alimentary tract Cases of gastro intestinal disorders and anæmia have been classified under those headings A comparison was also made, in the present investigation, of the response in a given individual with the two types of test meals, namely 'gruel oatmeal' and 'alcohol test meal' Twenty nine cases apparently healthy were given both types of meal at an interval of a few days

### *Comparative results with alcohol and gruel test-meals*

The use of the alcohol test-meal in gastric analysis is a comparatively recent procedure, while gruel has been in use for over 20 years In spite of the many technical advantages in the use of alcohol as a test-meal, there are still many clinicians who wonder how far alcohol is preferable to gruel as a stimulant to gastric mucosa It has been our experience that on a good many occasions when the patient showed poor or no response to the alcohol test-meal, clinicians have insisted on a repetition of the test with gruel This showed clearly a sceptic attitude towards the value of the alcohol test-meal Therefore, in the course of present investigation, 29 cases were given both alcohol and gruel on different occasions before a gastric analysis was done

The gruel test-meal has a number of disadvantages for instance, the patient's difficulty in swallowing such a bulky tasteless meal, the difficulty which the operator always experiences in drawing the samples and the time taken in filtering the various specimens before they are fit for chemical analysis Napier *et al* (*loc cit*) thought that a pint of any fluid is sufficient to dilute gastric juice appreciably and to lower the readings in the first hour The alcohol test-meal which consists of 100 c c of 7 per cent alcohol obviates all these disadvantages The 'meal' takes no time in preparation, it can be stocked for use when required, it can be passed directly into the stomach through Ryle's tube, little or no dilution of gastric juice takes place, the specimens can be drawn in bulks of 5 c c or 10 c c without

any risk of the holes of the tube being blocked, the specimens obtained are clear and can be used for chemical analysis without any preliminary filtration. Alcohol has been found to be a good stimulant to the gastric mucosa. The only objection raised against the use of alcohol as a test-meal is that it is not a natural stimulant for Indian patients but it can be seen that the gruel test meal is also not a natural gastric stimulant.

### TECHNIQUE

In the present series all the 121 cases were given 100 c.c. of 7 per cent alcohol. No preliminary preparations were made. The patient had his usual meal on the preceding evening. All tests were done in the forenoon on an empty stomach.

Analysis of the various specimens was done by titration with N/20 caustic soda. The results were expressed on the basis of the amount of N/10 NaOH required to neutralize the acid free and total present in 100 c.c. of gastric juice at a given moment. The reason for using N/20 NaOH was that by using a weaker solution the chances of error in titration are lessened and more accurate results are likely to be obtained.

Indicators used were the usual Topfer's reagent (0.5 per cent solution of dimethylamino benzene in alcohol) and 1 per cent solution of phenolphthalein in alcohol.

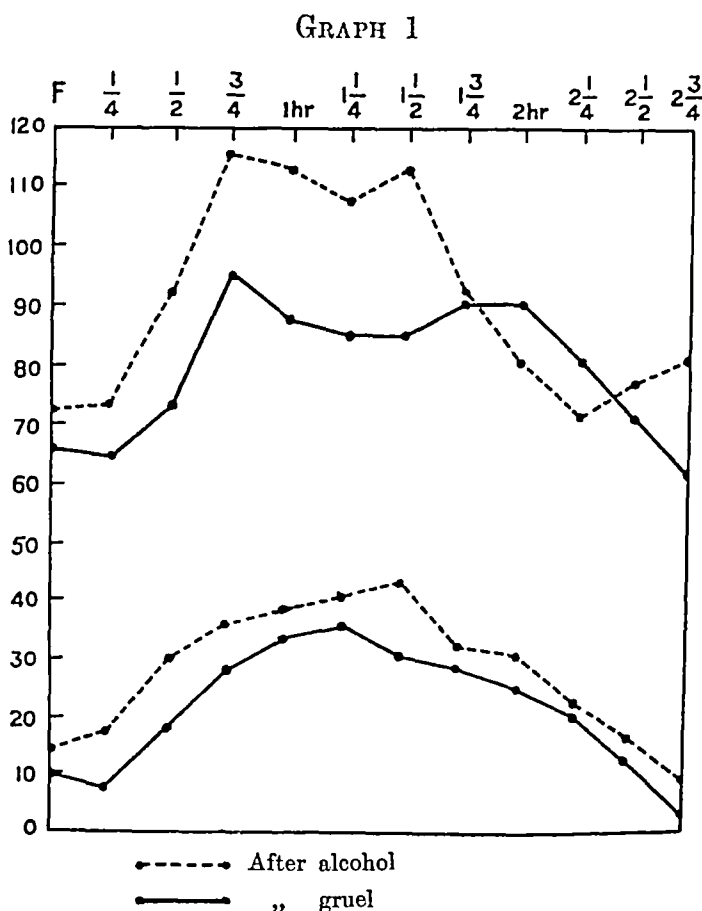
Only free acidity will be considered. Total acidity was also estimated, but as in almost all cases it runs more or less parallel with free acidity no reference will be made to total acidity.

Table I gives the mean acidity figures (expressed as usual in terms of N/10 NaOH per 100 c.c. of stomach contents) in 29 cases arranged according to 15-minute interval after removal of fasting contents —

TABLE I

Time of withdrawal of specimen in hour	Mean average with alcohol	Mean average with gruel	Maximum acidity with alcohol	Maximum acidity with gruel
Fasting	13.6	12.4	72	66
$\frac{1}{4}$	15.3	9.4	72	64
$\frac{1}{2}$	30.5	18.7	94	72
$\frac{3}{4}$	36.9	28.4	115	96
1	37.9	32.4	113	88
$1\frac{1}{4}$	38.0	33.4	108	86
$1\frac{1}{2}$	38.4	30.5	112	86
$1\frac{3}{4}$	31.7	30.5	90	90
2	31.5	25.3	80	90
$2\frac{1}{4}$	21.1	20.6	70	80
$2\frac{1}{2}$	16.1	12.1	75	70
$2\frac{3}{4}$	7.7	4.1	80	60

Mean and maximum acidity figures after the gruel and the alcohol test-meals are plotted in Graph 1 —



Showing mean and maximum acidity after gruel and alcohol test-meals in 29 cases

The time of first appearance of hydrochloric acid and the time of attainment of highest peak of acid were also studied with two types of meals. It was found out that both types of meals produce an almost similar mean average curve. If anything, alcohol appears to be a better gastric stimulant for the following reasons —

1 Maximum response with alcohol ranges between 70 and 115, while with gruel it ranges between 60 and 96

2 In 66 per cent of the cases the first appearance of free acid occurs 15 minutes after administering the alcohol test-meal, while with gruel only 52 per cent cases show the appearance of acid after 15 minutes

3 With both types of meal the highest peak is reached in 80 per cent of cases by the end of  $1\frac{1}{2}$  hours

## ACID RESPONSE IN NORMAL INDIVIDUALS AFTER ALCOHOL MEAL

The alcohol test-meal was done on 75 patients. Care was taken to include in this series only cases who had no sign or symptom pertaining to a disturbance of the gastro-intestinal tract. All of them had good appetite, no pain preceding or following food, no history of hæmatemesis or melæna and no vomiting. Cases of anæmia were excluded from this series.

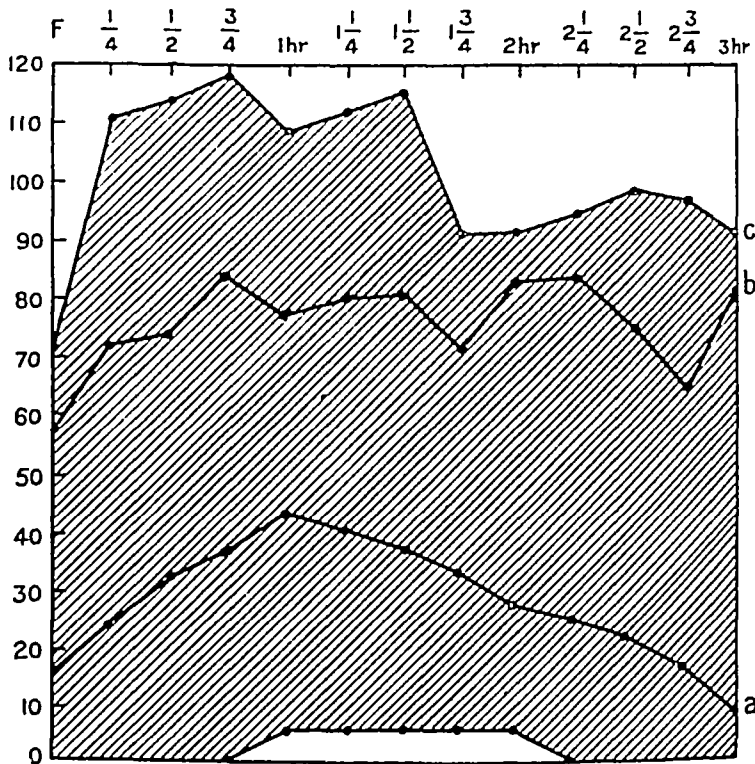
Table II shows the mean and maximum acid secretion after alcohol test-meal in 75 normal cases —

TABLE II

Time of sample in hour	Mean acidity figures	Maximum acidity figures (all cases)	Maximum acidity figures (88 per cent cases)
Fasting	14.2	72	58
$\frac{1}{2}$	21.3	110	72
$\frac{1}{2}$	31.6	112	72
$\frac{1}{2}$	36.2	118	84
1	40.0	108	72
$1\frac{1}{2}$	37.7	110	80
$1\frac{1}{2}$	36.8	112	80
$1\frac{1}{2}$	33.6	90	70
2	28.0	90	85
$2\frac{1}{2}$	25.8	92	85
$2\frac{1}{2}$	19.5	96	75
$2\frac{1}{2}$	14.2	95	62
3	7.2	90	80



GRAPH 2



After Mangalik and others, U P

Showing acid response in 75 cases after alcohol test meal

a—Mean acidity

b—Maximum acidity in 88 per cent cases

c—Maximum acidity in 100 per cent cases

Table II and Graph 2 bring out the following points —

1 Absence of an initial fall of acidity, which has been observed by most workers Rao (*loc cit*) and Napier *et al* (*loc cit*) found an initial fall of acidity soon after giving the meal in their normal cases. In this series, there was no initial fall of acidity. Looking at the individual figures of acidity, 15 minutes after giving the test-meal, it is found that out of 75 cases, 21 show an initial fall, while the others do not show such fall. Some cases do not show any free acid in the fasting specimen, while the first specimen taken after 15 minutes shows a good acid response. It appears that this absence of initial fall of acidity is due to alcohol acting as a powerful stimulant bringing forth good acid response which neutralizes the diluting effect of 100 c.c. of alcohol.

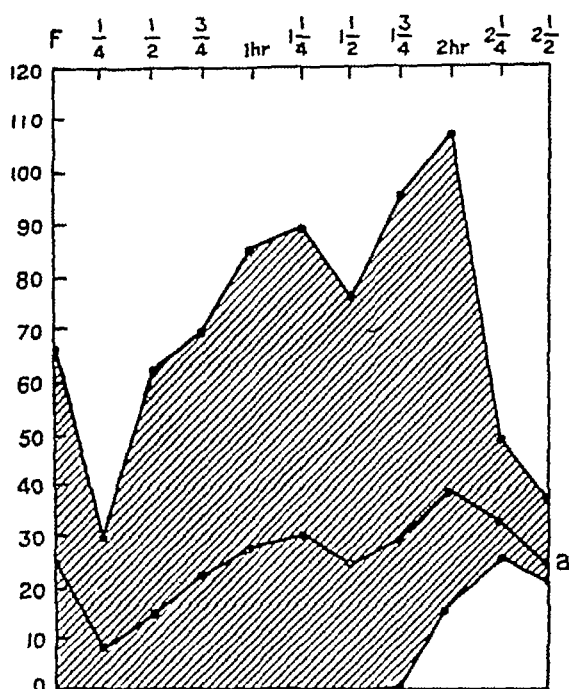
2 Some normal individuals show marked hyperchlorhydria. Nine cases showed acidity ranging over even the high normal limits classified by Napier

*et al* (*loc cit*) and Bell (1922), and reaching such high figures as 112. This is all the more significant, when it is realized that none of these patients had any evidence of disturbance pertaining to gastro-intestinal tract

### COMPARISON WITH OTHER WORKERS' RESULTS

Graphs showing gastric acidity, have been published by Napier *et al* (1935, 1938) in Indians in Calcutta and Bengali males. Rao (*loc cit*) published 'a com-

GRAPH 3



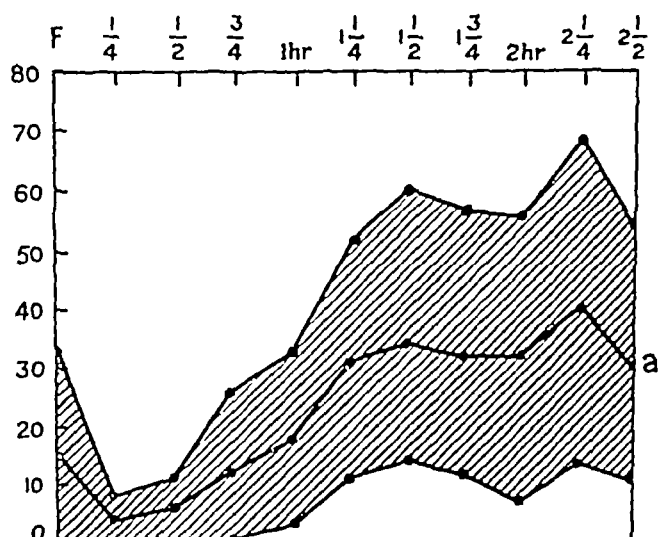
After Rao (1937), Madras

Showing range of acidity in 100 Madras Indians tested with gruel

a—Composite curve of mean acid values.

posite curve of acid values' and graphs 'showing variations in acidity of gastric juice' in Madras Indians. We reproduce below the graphs of these workers as also that of Ryle (1926) on English subjects (Graphs 3, 4, 5 and 6). The number of cases in each series as well as the nature of test-meal used has been mentioned below each graph.

GRAPH 4

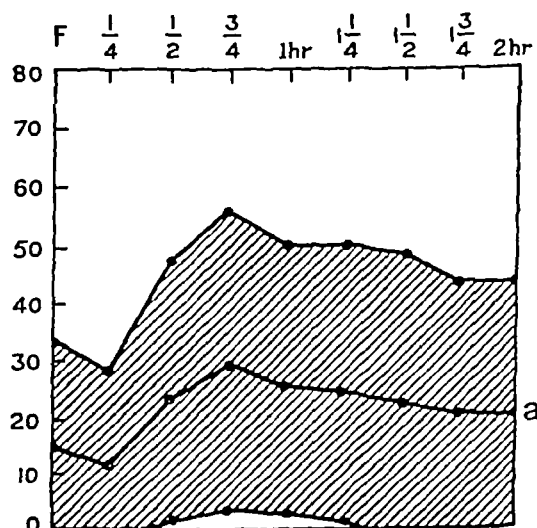


*After Napier and Das Gupta (1935), Indians  
in Calcutta*

Showing range of acidity in 14 cases tested at Calcutta  
with gruel

*a*—Mean acidity curve

GRAPH 5

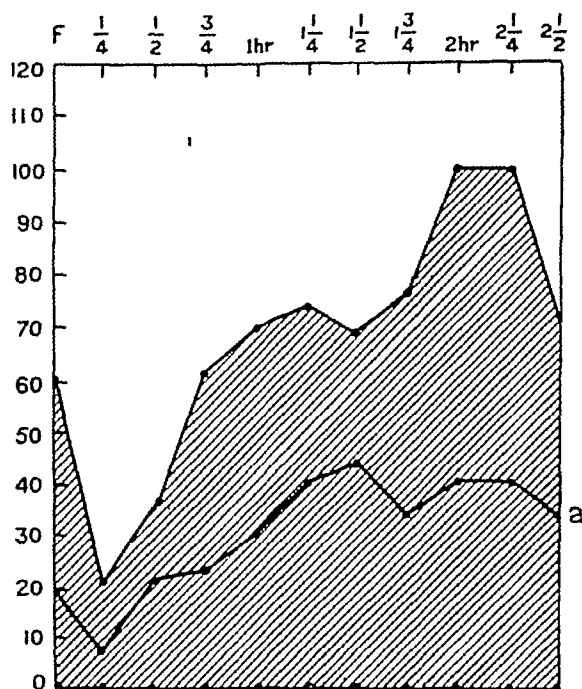


*After Napier et al (1938) Bengali males*

Showing range of acidity in Bengali males (89  
cases) with alcohol test-meal.

*a*—Mean acidity curve

GRAPH 6



*After Ryle (1926), England*

Showing range of acidity in 100 Englishmen tested with gruel

a—Range in 80 per cent cases

A comparative perusal of these graphs shows —

1 The mean fasting acidity in our series is 14.2, in Indians in Calcutta 15 (Napier and Das Gupta, *loc cit*), in Bengali males 14.6 (Napier *et al*, *loc cit*) and in Madras Indians 22 (Rao, *loc cit*)

2 The range of initial acidity shows marked differences. In Ryle's series, it ranges from 0 to 23 in 80 per cent of cases and from 0 to 62 in all cases. In Rao's series, it ranges from 0 to 50 in 80 per cent of cases, and 0 to 65 in all cases. In Napier's two series it ranges from 0 to 35. In our series, the range of initial acidity is higher—0 to 58 in 88 per cent of cases, and 0 to 72 in all cases. On the whole, the range of initial acidity seems to be on the higher side in these provinces, and more closely comparable to Indians in Madras.

3 Comparison of maximum acidity can be best seen from the following table —

Series	Cases, per cent	Maximum acidity	Time in hour
Our series (alcohol)	88	85	2
	100	118	$\frac{3}{4}$
Ryle (1926) (gruel)	80	47	$1\frac{1}{2}$
	100	100	2
Rao (1937) (gruel)	80	61	$1\frac{1}{2}$
	100	108	2
Napier and Das Gupta (1935) (gruel)	100	70	$2\frac{1}{4}$
Napier <i>et al</i> (1938) (alcohol)	100	58	$\frac{3}{4}$

On the whole, maximum acidity seems to be higher in these provinces

4 Mean acidity curves could also be compared on the same basis —

Series	Mean fasting	Mean highest	Time in hour
Our series (alcohol)	14.2	40	1
Rao (1937) (gruel)	22	42	2
Napier and Das Gupta (1935) (gruel)	15	40	$2\frac{1}{4}$
Napier <i>et al</i> (1938) (alcohol)	15	30	$\frac{3}{4}$

No mean acidity curve has been worked out by Ryle

The highest mean acidity of this series seems to be very close to that of gruel-tested cases of Rao (*loc cit*) and Napier and Das Gupta (*loc cit*) though the meals used were different. The highest peak, however, is reached one hour earlier in alcohol-tested cases. Comparing alcohol-tested cases of Napier *et al* (*loc cit*) in Bengal with ours, we find mean acidity in the United Provinces to be on the higher side.

5 The general contour of mean acidity curve of our series differs from those of other workers in India in having (a) no initial fall of acidity, (b) earlier

maximum peak, both differences being attributable to alcohol furnishing better gastric stimulation

#### CLASSIFICATION OF NORMAL CASES ACCORDING TO THEIR ACID RESPONSE

Bell (*loc cit*) established arbitrarily certain groups according to the range of maximum acid response showed by individuals Napier *et al* (*loc cit*) also adopted an arbitrary grouping to classify their cases Table III gives the classification given by these authors —

TABLE III

Groups		Napier's c c	Bell's, c c
I	Hyperchlorhydria	Over 65	Over 60
II-a	High normal (H)	Over 45	Over 30
II b	Average normal (M)	25 to 45	Under 30
II c	Low normal (I)	Under 25	Under 20
III	Hypochlorhydria	Under 10	Under 10
IV	Achlorhydria	No free HCl	No free HCl

Napier has further subdivided group IV (achlorhydria) into —

- A Tested with histamine
- A<sub>1</sub> With acid response
- A<sub>2</sub> Without acid response
- B Not tested with histamine

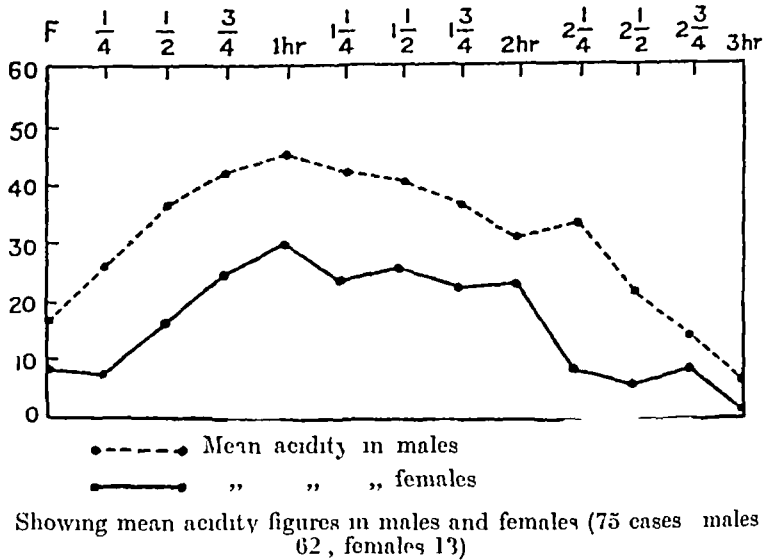
It will be seen that the limits fixed by Napier are distinctly higher than those of Bell The difference does not seem to be material as both groupings are arbitrary in nature We propose to classify our cases according to the groupings suggested by Napier It will give us an added advantage of being able to compare our findings with that of Napier's and to see if any significant difference is observed in the two series

*Acid response in Hindus and Mohammedans* — This was compared by studying the distribution of cases of both communities in various acid groups No significant difference could be brought out It was noted, however, that in both communities, the number of isochlorhydries is about 70 per cent

*Gastric acidity in the two sexes* — Graph 7 gives the mean acidity figures in males and females It shows a higher mean acidity in males Analysis of data obtained also showed that hyperchlorhydria is twice as common in men as in women The incidence of achlorhydria was found to be equal in the two sexes

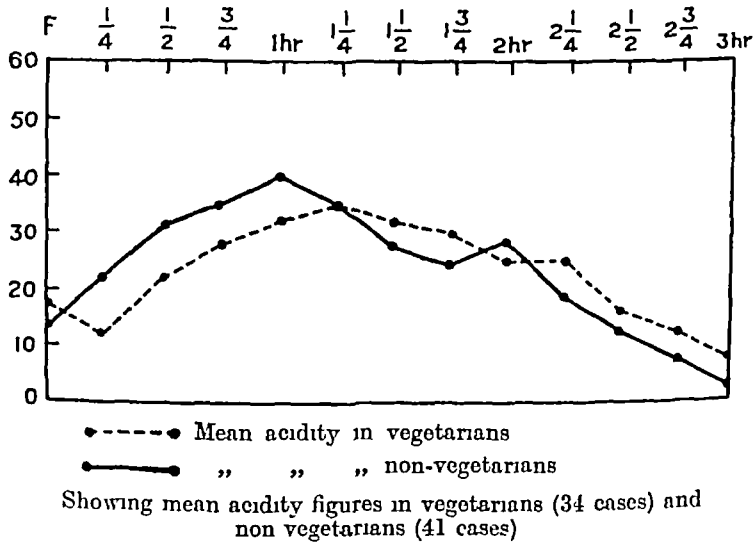
Napier *et al* (*loc cit*) found hyperchlorhydria three times as common amongst males as in females

GRAPH 7



*Influence of diet on gastric acidity*—Acid response in vegetarians and non vegetarians was studied and no difference was found. This is not surprising in view of the fact that this distinction is more apparent than real, both living on more or less the same type of staple diet, except that the non-vegetarians take meat occasionally. Graph 8 brings out the exact similarity in the acid response of vegetarians and non-vegetarians.

GRAPH 8



*Influence of age on gastric acidity*—The distribution of cases (males and females) into various acid groups according to their age was studied. It was found that with the increase of age the incidence of hyperchlorhydria increases, while in the age group of 20 years there were 26 per cent hyperchlorhydrics, at the age of 40 years the incidence increased to 35 per cent. Conversely, if the low acidity groups are taken together, their incidence decreases with increasing age group. These findings are in complete agreement with those of Napier *et al* (*loc cit*) who also found increase of hyperchlorhydrics with increasing age.

*Time of first appearance of hydrochloric acid*—This was studied in 70 cases, as 5 cases showing complete achlorhydria have been excluded. In 88 per cent cases, hydrochloric acid appeared within half an hour of test-meal. This compares well with findings of other workers. Ryle found the first appearance of acid within half an hour in 75 per cent of their cases. Gupta (*loc cit*) found 64 per cent of his normal cases showing presence of hydrochloric acid in the first half hour. Bhatt (*loc cit*) found first appearance of hydrochloric acid in 64 per cent of cases during the same period. The higher percentage of persons showing earlier acid response in our series is due, in all probability, to a better gastric stimulation by alcohol.

*Time of 'acid peak'* in 70 cases on a percentage basis, was found to be as follows—

- (i) Twenty-seven per cent of cases show maximum acid peak at the end of one hour
- (ii) Fifty-one per cent of cases show acid peak from one to one and a half hours

Table IV gives a comparison of time of maximum 'acid peak' according to various workers—

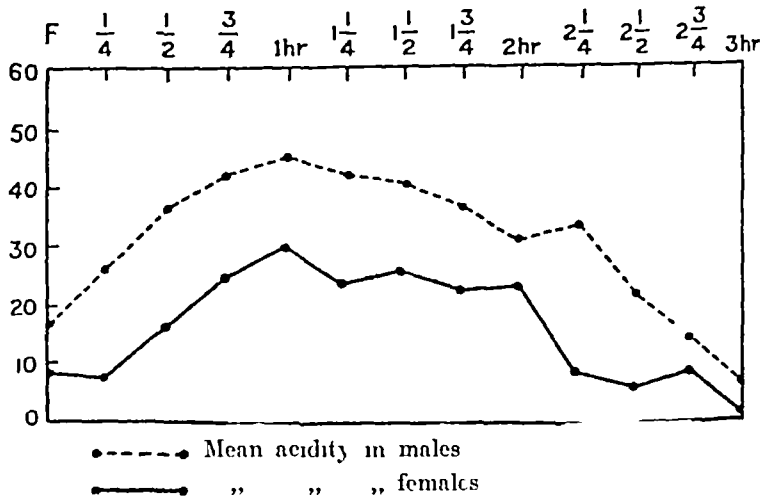
TABLE IV

Workers	Meal used	Time of maximum acid response in highest percentage in hour
Ryle (1926)	Gruel	1½
Bhatt (1930)	,	1½
Gupta (1933)	„	1½
Napier <i>et al</i> (1938)	Alcohol	¾
Our series	,	1



Napier *et al* (*loc cit*) found hyperchlorhydria three times as common amongst males as in females

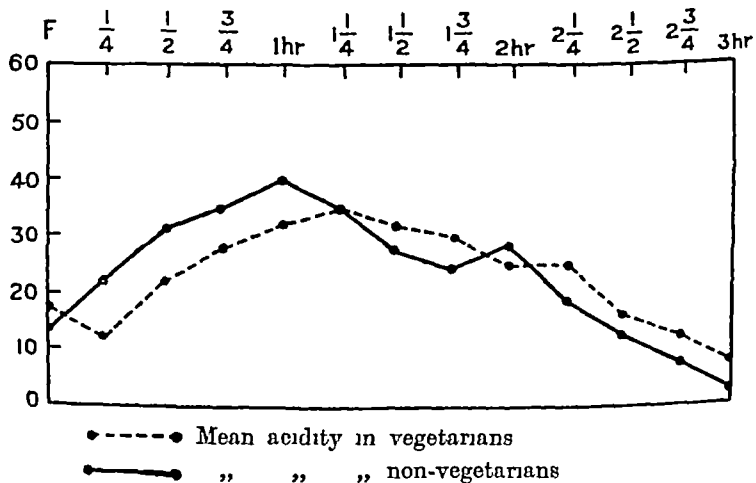
GRAPH 7



Showing mean acidity figures in males and females (75 cases males  
62 females 13)

*Influence of diet on gastric acidity*—Acid response in vegetarians and non vegetarians was studied and no difference was found. This is not surprising in view of the fact that this distinction is more apparent than real, both living on more or less the same type of staple diet, except that the non-vegetarians take meat occasionally. Graph 8 brings out the exact similarity in the acid response of vegetarians and non-vegetarians.

GRAPH 8



Showing mean acidity figures in vegetarians (34 cases) and  
non vegetarians (41 cases)

although there are some people who do not favour the use of a toxic drug like histamine, the consensus of opinion is that histamine furnishes an excellent gastric stimulus, whereby it can be demonstrated that true 'achlorhydria' or 'achylia gastrica' is a comparatively rare occurrence

In the present series of 121 cases the distribution of achlorhydria was as follows —

	Total number of cases	Number of cases showing achlorhydria
1 Normal	75	5
2 Diseases—	46	12
(a) anæmias	23	9
(b) gastro intestinal lesions	23	3

Table V shows the distribution of cases showing achlorhydria —

TABLE V

	Total number of cases	Number of cases showing achlor hydria	No acid response after histamine	Acid response after histamine	Could not be tested with histamine
Normal	75	5	2		3
Anæmia	23	9	3	4	2
Gastro intestinal lesion	23	3	1	1	1
TOTAL	121	17	6	5	6

Achlorhydria has been subjected to a critical review by Oliver and Wilkinson (1933). They quote the figures given by other authors as follows —

'Bennet and Ryle (1926) found achlorhydria in only 4 per cent cases of their normal series. Dahl Iverson saw 11 per cent with achylia in 128 normal persons. Baird Campbell and Hern observed 1 case out of 57 students while Apperley and Semens found 8 in a group of 90 similar subjects. Rehfuess (1929) and Chenev (1926) are sceptical about the occurrence of normal achlorhydria and they believe that it may be the forerunner of several vital ailments. Bloomfield and Keefer believe that occurrence of achlorhydria is quite common without clinical significance.'

In Napier's series the total incidence of achlorhydria is 13 out of 209 cases, which is less than 6 per cent. In the present series, 17 cases showed achlorhydria

with alcohol Six cases could not be tested with histamine while 11 cases were subjected to histamine test, out of which 5 showed the presence of hydrochloric acid If the response to histamine in 6 untested cases be considered to be 45 per cent as in 11 tested cases, the incidence of achlorhydria is 9.3 in 121 cases, which is about 7.7 per cent By the same method of deduction, one finds that in the present series of 75 normal individuals the incidence of histamine-fast achlorhydria will work out to 6 per cent

From the above analysis of achlorhydria, it seems that cases of apparent achlorhydria, when tested by histamine, show acid response in about 50 per cent In other words, the incidence of true achlorhydria which should alone be taken to mean achlorhydria is about 50 per cent of apparent achlorhydria It seems unjustifiable to label a case as 'achlorhydria' unless subjected to the histamine test-meal

#### GASTRIC RESPONSE IN THE DISORDERS OF GASTRO-INTESTINAL TRACT AND ANÆMIAS

In all 46 cases were examined and they could be divided into two groups —

*Group A* —Cases in which predominant symptom was disturbance of the gastro-intestinal tract (23 cases)

*Group B* —Cases in which anæmia was the predominant condition This group was fully investigated as regards the blood picture according to modern hæmatological technique and comprised 23 cases

These groups are dealt with separately

*Group A* —Cases of *gastro-intestinal disorder* —These cases could be distributed into the various acid groups laid down before as follows —

TABLE VI

Diagnosis	Number of cases	Hyperchlorhydria	ISOCHLORHYDRIA			Hypochlorhydria	ACHLORHYDRIA		
			H	N	L		A	A <sub>1</sub>	B
Peptic ulcers	14	8	4	1	1				
Sprue	4	1	1				1	1	
Growths	2				1				1
Undiagnosed	3	2			1				

The majority of patients with peptic ulcer showed either hyperchlorhydria or isochlorhydria of the high normal type Active peptic ulcers may be compatible with normal acid response, but not compatible with hypochlor- or achlorhydria

Gastric acidity in sprue was found to be variable. The test-meal showed hyperchlorhydria in one case, isochlorhydria in another and achlorhydria in two one of which proved to be histamine-fast. Fairley (1930) found free hydrochloric acid with ordinary test-meals in 30 cases out of 44 in the series. Vaughan (1936) found 5 out of 18 cases having histamine-fast achlorhydria. The achlorhydrics recovered the power of secreting acid during remission. Gupta (*loc cit*) found 2 cases of sprue with normal acid response, one showing hypochlorhydria and 3 showing achlorhydria—not tested with histamine.

*Group B—Gastric acidity and the anæmias*—In this series 23 cases were examined and they have been classified into the various acid groups as given in Table VII—

TABLE VII

Type of anæmia	Number of cases	Hyperchlorhydria	Isochlorhydria	Hypochlorhydria	ACHLORHYDRIA		
					Histamine fast	With acid response to histamine	Not tested with histamine
I Macrocytic hyperchromic	12						
(a) Pernicious anæmia	1	—	—	—	1	—	—
(b) Tropical macrocytic anæmia	11	—	4	3	—	4	—
II Microcytic hypochromic	11						
(a) Ankylostomiasis	7	2	3	—	1	—	1
(b) Bleeding piles	1	—	1	—	—	—	—
(c) Cause not known	3	—	1	—	1	—	1

*Gastric acidity in macrocytic anæmias*—Only one case satisfying the cardinal features of Addisonian pernicious anæmia was met with in this series which showed histamine-fast achlorhydria.

Eleven cases of tropical macrocytic anæmia were examined. Four cases showed isochlorhydria, 3 hypochlorhydria and 4 showed achlorhydria with alcohol test-meal, which on subsequent examination with histamine showed secretion of free hydrochloric acid. One case out of these showed blood picture of macrocytic hyperchromic anæmia, achlorhydria on three occasions with the alcohol meal and signs of involvement of the posterior and lateral columns. A diagnosis of Addisonian pernicious anæmia had to be given up, when on subsequent examination

with histamine, it showed free hydrochloric acid. The patient responded to treatment with Marmite in a marked way. The case is interesting in so far as it shows neurological manifestations in a case of tropical macrocytic anæmia. It brings out very clearly that apparent achlorhydria is not to be accepted at its face value. No case is to be labelled as achlorhydric unless confirmed with histamine.

A proper gastric analysis is very valuable in the differential diagnosis of Addisonian pernicious anæmia and tropical macrocytic anæmia.

Wills (1931) introduced the term 'tropical macrocytic anæmia'. She distinguished this disease from true Addisonian pernicious anæmia by (a) the presence of free acid in gastric secretion (b) a negative indirect van den Bergh reaction, (c) an increase in the white cell count and (d) absence of neurological symptoms. In our experience, as also Napier's (*loc cit*), the results of van den Bergh reaction are variable in tropical macrocytic anæmia. While some cases show a negative van den Bergh reaction, others, perhaps in larger number, show a positive indirect reaction. Neurological manifestations are distinctly rare in tropical macrocytic anæmia. Again, all cases of pernicious anæmia do not show evidence of involvement of posterior and lateral columns of the cord.

It seems reasonable to conclude, therefore, that the one reliable guide to distinguish between true Addisonian pernicious anæmia and tropical macrocytic anæmia is the presence of histamine-fast achlorhydria in the former. It stresses, once again, the importance of the histamine test-meal in cases of achlorhydria.

*Gastric acidity in microcytic hypochromic anæmia*—In all, 11 cases were investigated in this group. In 7 the cause of anæmia was infection with ankylostomes and in 4 the cause of anæmia could not be determined. The distribution of these cases was as follows—

Hyperchlorhydrics (above 65) 2 cases

Isochlorhydrics (from 45 to 10) 5 cases

Hypochlorhydrics (from 10 to 0) *nil*

Histamine-fast achlorhydrics 2 cases

Achlorhydrics without histamine test 2 cases

It would seem that the relation of the gastric acidity and hypochromic microcytic anæmia is very variable. The cases seem to be nearly equally distributed in the various acid groups. The fact worth stressing seems the presence of histamine-fast achlorhydria in 2 of these cases, one being a case where ankylostomiasis was the ætiological factor, while in the other no ætiological factor could be discovered. The relation of histamine-fast achlorhydria and microcytic hypochromic anæmia is very well known. In cases where no apparent cause for this type of anæmia is discoverable and the patient has histamine-fast achlorhydria, the usual diagnosis made is idiopathic hypochromic anæmia (Witts, 1930, 1931). One of these 2 cases appears to belong to this group. In the other case, where ankylostomiasis was the causative factor, it is justifiable to postulate that achlorhydria aggravated the deficiency state, leading to anæmia. Achlorhydria

is well known to cause loss of appetite for iron-containing foods such as meat or green vegetables. The absorption of iron is probably diminished in the absence of free hydrochloric acid, and lastly to quote Witts (1931) 'achlorhydria is often associated with other signs of asthenic diathesis in which anæmia so readily occurs'

#### SUMMARY AND CONCLUSIONS

1 Fractional gastric analysis with alcohol as the test-meal has been done on 121 persons of the United Provinces

2 The alcohol test-meal and the gruel test-meal have been used on the same individuals in 29 cases. A comparison between the gastric response to alcohol and gruel meals respectively showed that alcohol is an equally good or perhaps a better and more powerful gastric stimulant

3 With alcohol as a test-meal the gastric curve is characterized by the absence of an initial fall in acidity after giving the test-meal. A possible explanation of this finding has been discussed

4 Normal acid response in Indians in these provinces has been compared to Indians residing in the provinces of Madras and Bengal, as also with the acid response in Englishmen as reported by Ryle

5 Gastric analysis on 75 normal individuals showed that, on the whole, the acid response in Indians of these provinces shows higher initial and maximum acidity as compared with Indians in Bengal or Madras

6 The distribution of the normal cases has been classified according to (a) community, (b) sex, (c) dietetic differences, and (d) age

(a) There is no significant difference in the gastric response of Hindus and Mohammedans

(b) Hyperchlorhydria seems to be more common in males

(c) Daily diet does not influence the gastric response as judged by the study of gastric acidity in vegetarians and non-vegetarians

(d) Increase of hyperchlorhydria with increase in age has been noticed

7 In 90 per cent cases the time of first appearance of hydrochloric acid seems to be within first half an hour of test meal

8 Maximum 'acid peak' in the majority of cases is reached within one hour

9 The average fasting stomach contents work out to 30 c.c.

10 Incidence of achlorhydria in normal individuals as well as in diseased persons is discussed. The incidence of histamine-fast achlorhydria in 75 normal cases works out to about 6 per cent

11 A study of gastric acidity on 46 patients, suffering from gastro-intestinal disturbances and anæmias has been made. Fourteen cases of peptic ulcer

4 cases of sprue and 23 cases of various types of anæmia have been studied. The type of response in various conditions has been discussed.

12 The use of histamine in cases of apparent achlorhydria is stressed, especially as it seems the only reliable method of distinguishing tropical macrocytic anæmia from true Addisonian pernicious anæmia.

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## A MOUSE-PROTECTION TEST FOR THE ASSAY OF TETANUS TOXOID

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[Received for publication, February 24, 1942]

FOR the biological assay of a toxoid two methods suggest themselves (1) Measurement of the tolerance to toxin of selected test animals immunized with the toxoid or (2) measurement of the antitoxin content resulting from such an immunization. The latter method has been laid down in the Regulations under the Therapeutic Substances Act, 1939, for determining the potency of tetanus toxoid for human immunization. There is, however, no experimental evidence in literature showing the comparative value of the two methods. The following experiments were, therefore, undertaken to elucidate this point —

### METHODS

The two methods of assay of tetanus toxoid employed were as follows —

*Determination of immunizing power of a toxoid by testing the tolerance of immunized animals for toxin* — White mouse is employed as the test animal. An amount of prophylactic equivalent to half the human dose is injected subcutaneously into more than 30 white mice of 23 g to 27 g body-weight. The animals are tested for immunity to tetanus toxin 4 weeks after the injection of the toxoid, by subcutaneous injection of twenty definite lethal doses of toxin. By a definite lethal dose is meant a dose of toxin which kills all the 4 mice under test, while the next lower dose, which is two-thirds the preceding dose, fails to kill all of them. The toxoid is held to be sufficiently potent if at least one-third of the total number of mice in the herd survives—the duration of observation being limited to 5 days. The test is named as the *mouse-protection test* for convenience of expression.

*Determination of immunizing power of a toxoid by testing the antitoxin content of serum of immunized animals* — This test laid down in the Therapeutic Substances Act was carried out as follows. An amount of toxoid five times the adult human



dose is injected subcutaneously into more than 9 guinea-pigs of 250 g to 350 g body-weight. Six weeks after the injection the animals are bled and the sera tested for their antitoxin content, in terms of International units. The toxoid is held to be sufficiently potent if at least two-thirds of the total number of guinea pigs show not less than 0.1 International unit of antitoxin per c.c. of serum. The test is named as the *antigenicity test in guinea-pigs* for convenience of expression.

#### EXPERIMENTAL OBSERVATIONS

*Comparison of the two tests*—For this purpose, three different batches of toxoids showing different mouse-protection value on preliminary test were selected for the experiment. The results are shown in Table I. The rate of survival of mice immunized with 0.5 c.c. of toxoid against twenty definite lethal doses of toxin, is expressed as the mouse-protection value. Similarly the proportion of guinea pigs showing 0.1 International unit or more of antitoxin per c.c. of serum after injection of 5 c.c. of toxoid, is expressed as the antigenic value in guinea-pigs.

TABLE I

*Correlation of the mouse-protection value and the antigenic value in guinea-pigs*

Toxoid batch number	Mouse-protection value	ANTIGENIC VALUE IN GUINEA-PIGS	
		1st test	2nd test
37	4/30	6/10	8/12
44	9/31	10/11	8/9
34C	13/31	10/10	9/10

The result shows roughly that toxoid batch 37, which fails to satisfy the mouse test, barely passes the antigenicity test in guinea-pigs in the second test only. Toxoid batch 44, which just fails to satisfy the mouse test, and toxoid batch 34C, which satisfies the test easily, pass the guinea-pig test satisfactorily. It seems, therefore, that a toxoid satisfying the mouse-protection test as outlined here, is likely to satisfy in every case the antigenicity test in guinea-pigs as laid down in the Regulations under the Therapeutic Substances Act. Thus, the method seems to be quite useful as a means of determining that the prophylactic satisfies some minimum requirement of antigenicity. The numerical figures indicating values obtained on testing the toxoids of batch 37 and batch 44 seem to show that the mouse test is more severe than the guinea-pig test. When the wide variations amongst animals in response to toxoid injection is, however, taken into consideration the real significance of such numerical figures becomes very doubtful.

*Comparison of the mouse with the guinea-pig as a test animal suitable for toxin-tolerance test*—Twenty-five guinea-pigs of about 300 g body-weight and the same number of mice of about 20 g body-weight were injected with 1 c c of toxoid subcutaneously. Four weeks later, 5, 7.5, 10 and 15 m l d of toxin, determined on animals of the same species and of weight expected to be reached by the animals after 4 weeks, were injected after dividing the above into four groups of 6 animals of each species, successively. Table II records the rates of survival of animals after 5 days' observation —

TABLE II

*Rates of survival after injection of toxin into animals immunized with toxoid*

Dose, m l d	SURVIVAL	
	Mouse	Guinea pig
5	5/6	5/6
7.5	3/6	4/6
10	3/6	5/6
15	1/6	1/5

It appears from the result of this experiment that the mouse responds almost as vigorously to tetanus toxoid as the guinea-pig, if no consideration is paid to the dose in relation to the body-weight. The importance of the dosage of the toxoid in relation to the body-weight is not well understood. In the official test guinea-pigs, which are about  $\frac{1}{25}$  the body-weight of man, are injected with only one-tenth the human dose in the two dose immunization method, and yet they do not produce a higher level of antitoxin than is produced in average man.

*Variation in response to toxoid of small groups of mice in a big herd*—This experiment was undertaken to determine the effect of the age of toxin on the measurements of the degree of immunity in mice immunized with tetanus toxoid. The results obtained were, however, interesting otherwise.

Mice were immunized with 1 c c of toxoid subcutaneously. Eight weeks later they were tested with two different toxins—batch 12 prepared 2 months before test and stored in the liquid state under toluol at 2°C, and batch 25, prepared only 10 days before test and stored under the same conditions. Both

the toxins were filtrates of 4 days' growth of *Cl tetani* in Robertson's cooked meat media containing 0.2 per cent glucose

Mice were grouped in sixes in each cage. Those in four such cages received the same number of mld. Four doses were injected: 3.75, 7.5, 11.25 and 15 mld. Survival rates after 5 days of observation are recorded in Table III —

TABLE III  
Rates of survival of immunized mice tested with different doses of toxin

Test dose, mld	BATCH 12		BATCH 25	
	Cage number	Survival	Cage number	Survival
3.75	1	3/6	17	3/6
	2	2/6	18	0/6
	3	4/6	19	2/6
	4	4/6	20	2/6
7.5	5	0/6	21	2/6
	6	1/6	22	3/6
	7	1/6	23	2/6
	8	1/6	24	1/6
11.25	9	1/6	25	0/6
	10	2/6	26	0/6
	11	2/6	27	2/6
	12	0/6	28	0/6
15	13	0/6	29	0/6
	14	1/6	30	1/6
	15	0/6	31	2/6
	16	2/6	32	0/6

The interval of 8 weeks was selected arbitrarily with the theoretical idea of allowing the immunity in rapidly responding and slowly responding animals to level up to some extent, in the former by retrogression and in the latter by progression.

It appears to be clear from this experiment that mice differ individually in their response to immunization with toxoid. Though the difference in the rates of survival between two groups of 24 mice at any particular level of the test dose

of toxin does not seem to be very significant, it is interesting to note that with a high dose of toxin, 15 mld for instance, the difference in the numerical figures of the rates of survival between two groups of 24 mice becomes *nil*. This result may be due to chance or due to the big dose of toxin, which may cover much of the difference between any two groups of 24 mice by eliminating all but the highly immunized mice forming possibly a more constant population in a herd.

The two toxins used for the test seem to have no effect in causing any difference, because the survival rate is in favour of one toxin at one level of test dose, while it is in favour of the other toxin at the next level.

*Variation in the result on repetition of the test*—In order to check the soundness and dependability of the mouse test, it is necessary to test a toxoid by this method a number of times and compare the results to determine the probable range of variation in the result of the test.

For this purpose, toxoid batch 34C was selected for the experiment. In all three tests were done. Results are recorded in Table IV—

TABLE IV

*Different values of toxoid obtained on repetition of the mouse-protection test*

Number of test —		1	2	3
Group	Date of immunization —	26-4-41	26-6-41	12-7-41
	Date of trial —	22-5-41	24-7-41	9-8-41
Group A	Number of mice immunized	18	18	16
	Number of mice dying before trial	3	1	0
	Survival rate of mice tested	6/15	8/17	6/16
Group B	Number of mice immunized	18	18	16
	Number of mice dying before trial	2	0	0
	Survival rate of mice tested	7/16	9/18	9/16

The result of this experiment indicates that a particular toxoid shows a fairly constant immunizing value when it is repeatedly tested by the mouse protection test

*Correlation of the mouse-protection value of a toxoid and its antigenic value in man*—Two toxoids, batch 37 and batch 34C, with mouse-protection values of 4/30 and 13/31 respectively, were selected for the experiment. Two doses of 1 c.c. each were injected deep subcutaneously at about 6 weeks' interval. Samples were collected about 4 weeks after the last injection and the sera titrated at 0.2 International unit level (Table V)

TABLE V

*Result of immunization of man with two toxoids of different mouse-protection values*

Number of volunteers	Interval between 1st and 2nd injections, days	Interval between 2nd injection and sampling, days	Batch of toxoid injected	Whether passed the 0.2 unit level or not
1	52	34	34C	No
2	52	34	37	Yes
3	52	34	34C	Yes
4	52	34	37	Yes.
5	52	34	34C	Yes
6	52	34	37	Yes
7	52	34	34C	Yes
8	52	34	34C	Yes.
9	52	34	37	No
10	52	34	34C	Yes
11	52	34	34C	Yes.
12	52	34	37	No
13	52	34	34C	Yes
14	52	34	37	Yes
15	52	34	34C	No

TABLE V—*concl'd*

Number of volunteers	Interval between 1st and 2nd injections, days	Interval between 2nd injection and sampling, days	Batch of toxoid injected	Whether passed the 0.2 unit level or not
16	52	34	34C	Yes
17	54	32	37	No
18	52	34	37	No
19	52	33	37	No
20	52	33	37	No
21	52	33	34C	Yes.
22	52	33	37	Yes
23	52	33	34C	Yes
24	52	33	37	No
25	52	33	34C	No
26	52	33	37	Yes.
27	52	33	34C	Yes.
28	52	34	34C	No
29	52	34	37	No
30	52	34	34C	No
31	52	34	34C	Yes

The result briefly summarized shows that out of 17 men injected with two doses of toxoid, batch 34C, 12 passed the 0.2 International unit level, and out of 14 men injected with two doses of toxoid, batch 37, only 6 passed the 0.2 unit level.

The result thus shows in a rough way the difference in antigenic values of the two toxoids, which showed different mouse-protection values, though the figures as obtained on a small number of subjects are not very significant.

*Immunization of man with a toxoid satisfying the mouse test*—A mixture of toxoids was used for immunization. The mixture had a mouse-protection value of 12/31. Twelve men were immunized with two doses of 1 c.c. each at 41 days'.

interval Four weeks after the last dose samples of blood were collected and serum titrated in mice in terms of American units (Table VI)

TABLE VI

*Amounts of antitoxin produced in man immunized with a toxoid satisfying the mouse test*

Number	Units of antitoxin		Number	Units of antitoxin	
1	>0.5	<1.0	7	>0.1	<0.25
2	>0.1	<0.25	8	>0.25	<0.5
3	>0.1	<0.25	9	<0.05	
4	>0.05	<0.1	10	>0.1	<0.25
5	>0.25	<0.5	11	>0.25	<0.5
6	0.5		12	>0.5	<1.0

Ten out of 12 men showed a titre more than and only two less than 0.1 American unit (0.2 International units). This result supplies some additional evidence showing that the mouse-protection test can discriminate between a useful and useless toxoid.

#### DISCUSSION

The mouse-protection test was devised on the basis of the test for potency of diphtheria prophylactics laid down in the British Pharmacopœia, 1932. The immunizing dose which for diphtheria prophylactic is laid down to be 'five times the volume indicated as the adult dose' was reduced to one-tenth in proportion to body-weight of the mouse in relation to that of the guinea-pig. The test dose selected was twenty lethal doses of tetanus toxin. The idea was to differentiate sharply between the immune and the non-immune. Allowance was made for wide variation in receptivity of mice to tetanus toxoid by taking a result such as 10 surviving out of 30 animals tested as significant of sufficient potency.

Now that a test has been laid down in the Regulations under the Therapeutic Substances Act, the mouse test cannot be used as a final test for passing a toxoid for human use. Nevertheless, the test seems to be sufficiently reliable for differentiating between a useful and a useless toxoid. Thus, it may be applied for preliminary assays instead of the guinea-pig test, which is comparatively expensive in

time (at least 7 weeks) and animals at least 9 guinea-pigs for immunization and six times that number of mice for measuring the antitoxin content of the immunized animals at one level only, for according to Llewellyn Smith (1938) 'groups of 6 guinea-pigs or 6 mice for each mixture should result in an adequate precision of testing'

### SUMMARY

1 A method of biological assay of tetanus toxoid by testing the tolerance of immunized white mice for a dose of toxin is described

2 It is shown that a toxoid passing the mouse test also passes the test laid down in the Regulations under the Therapeutic Substances Act, 1939

3 Further studies on some of the different aspects of the mouse test elicited the following results —

- (a) The mouse is as suitable as the guinea-pig for toxin-tolerance test
- (b) Though the white mice differ widely in their response to immunization with toxoid, it seems a big dose of toxin, such as 15 mld or more, eliminates the individual variation by eliminating all excepting the highly immune individuals, if a large group of 24 individuals or more is used
- (c) The measurement of the potency of a toxoid with the mouse test gives repeatable results within very close limits
- (d) There seems to be a fair degree of correlation between the mouse-protection value of a toxoid and its antigenic value in man
- (e) A toxoid passing the mouse test can immunize man successfully

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## NUTRITIONAL REQUIREMENTS OF *PS PYOCYANEA*

BY

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NUTRITION is a basic factor profoundly influencing the life processes and unless the nutrient needs are adequately met an organism rapidly tends to undergo variations from the normal. In ascertaining the nutritional requirements of an organism simple synthetic media afford the best starting point. In these the introduction of new factors can be accurately controlled. This method has not been widely employed in the study of pathogenic bacteria. The aim of the present investigation was to construct a simple synthetic medium out of substances of known composition best suited for the profuse growth and pigment production of *Ps pyocyanea*, to examine further the minimum food requirements necessary for its growth and metabolism, to ascertain whether pyocyanus can produce pyocyanin (blue pigment) in synthetic media devoid of peptones, to establish whether or not magnesium ions stimulate the growth or pigment production and to find out the optimum pH, an important factor in the synthetic machinery of cell nutrition, for profuse growth and pigment production.

In the scale of pathogenicity *Ps pyocyanea* occupies only a low position. It is one of those organisms with which the parasitic mode of living has not become a permanent feature of the life cycle. Far from being a complete parasite, it can easily revert to a simple free-living existence independent of animal or plant life without any adaptative training. It is therefore, a comparatively easier task to

discover what chemical units must be supplied as raw materials from which to build up its nutritional requirements

*Experimental technique*—Throughout the investigation, the test-tubes used to grow the organism were all of the same size and of the same kind of glass and they were plugged with cotton-wool after cleaning thoroughly with sulphuric dichromate mixture. Finally, they were sterilized in the hot-air chamber at a temperature of 160°C for one hour. Into these tubes various test solutions were put by means of accurately graduated pipettes and afterwards they were once more sterilized. The chemicals used were Merck's (guaranteed) analytical reagents and the distilled water was ammonia free. The same platinum loop was used throughout for inoculations. Growth was observed macroscopically and the degree of it judged by the opacity. Duplicates were kept in every case. The percentage of ingredients employed was ammonium chloride 0.15, pot dihydrogen phosphate 0.1, sodium citrate 0.2, magnesium sulphate 0.02, ammonium sulphate 0.15, calcium lactate 0.2 and lactic acid (re-distilled, sp. g. 1.21) 1, all in sterile distilled water. These substances were accurately weighed in a chemical balance and proper dilutions were made under strict aseptic conditions. All possible combinations of these ingredients were embodied in a variety of media and made in bulk, and finally constant volumes of them put in sterile test-tubes, as mentioned above, and sterilized at 5-pound pressure in an autoclave for one hour.

It should be emphasized that scrupulous attention should be paid in the cleaning of glassware. This should be done with dichromate sulphuric acid mixture. Further, it was found that after every inoculation the platinum loop after flaming should be washed once or twice by dipping in the sterile distilled water. This procedure eliminated entirely the carrying over of extraneous salts that may encrust on the loop during the act of flaming. In the early stages of the investigation when this precaution had not been taken puzzling discrepancies in the results were often encountered.

Nine strains of *Ps pyocyanea* isolated from various pathological specimens received from the King George Hospital, Vizagapatam, and one from an infected fowl were selected for the investigation. Preliminary cultures of these strains were made separately in tubes each containing 5 c.c. of Dunham's peptone-water and incubated aerobically at room temperature. After 24 hours' incubation a loopful of the culture was inoculated into 5 c.c. of Koser's citrate solution in which the organism grows profusely. In the same fashion after every 24 hours, sub-inoculations were made into fresh Koser's tubes for 10 days. This was done to ensure that the inoculum was free from the last traces of peptone. Finally, a drop of the culture from the last Koser's tube (10th) was mixed with 10 c.c. of sterile distilled water. From this diluted culture, the several experimental synthetic media were inoculated by means of the same platinum loop. The tubes containing the cultures were all incubated aerobically at laboratory temperature—(approximately between 22°C and 32°C). Peptone-water controls were kept side by side with the test cultures.

TABLE I

## Growth at pH 7.0

Source of strain	Strain	1	2		3	4		5	6
		NH <sub>4</sub> Cl + sod citrate pH 7.0	NH <sub>4</sub> Cl + sod citrate + KH <sub>2</sub> PO <sub>4</sub>		NH <sub>4</sub> Cl + sod citrate + MgSO <sub>4</sub> ·7H <sub>2</sub> O, pH 7.0	NH <sub>4</sub> Cl - sod citrate + KH <sub>2</sub> PO <sub>4</sub> + MgSO <sub>4</sub>		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + sod citrate + KH <sub>2</sub> PO <sub>4</sub> , pH 7.0	Peptone water (control), pH 7.0
			Acid side.	pH 7.0		Acid side	pH 7.0		
Blood	K G H 1	—	G	GGG	—	GG	GGG	GGG (pellicle)	GGG } PPP }
"	" 2	—	"	"	—	"	"	" "	"
Ear swab	" 3	—	"	"	—	"	"	" "	"
Sputum	" 4	—	"	"	—	"	"	" "	"
Ascitic fluid	" 5	—	"	"	—	"	"	" "	"
Urine	" 6	—	"	"	—	"	"	" "	"
Liver pus	" 7	—	"	"	—	"	"	" "	"
Blood	" 8	—	"	"	—	"	"	" "	"
"	" 9	—	"	"	—	"	"	" "	"
Infected fowl	" 10	—	"	"	—	"	"	" "	"

Note — — = negative, no perceptible growth, G = slight growth, GG = good growth, GGG = profuse growth with pellicle formation GGG } = profuse growth and pigment production  
ppp }

From Table I it is found that media Nos 2, 4 and 5 are satisfactory for the production of profuse growth at neutral reaction of the media but are not

growth and pigment production it was 7.4. As pointed by Pandalai (1941), the organism produces a fair amount of alkali during growth in the synthetic media also and the final pH of the media was found to vary between 7.6 and 8. The latter limit may even be exceeded in the case of an occasional strain. A comparison of the results in column 2, Table III, with those of column 3, Table II, shows that sulphur stimulates pigment production. It may be supplied either as sulphate or sulphide.

From the results shown in Tables II and III it is clear that the addition of calcium lactate to media which do not help any pigmentary activity induces pigment production. The following experiment was performed to ascertain whether it was calcium or lactate or both the ions that were necessary for inducing pigmentary activity in such media —

TABLE IV

*Effect of lactic acid on growth and pigment production*

Strain		1		2		3		4
		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + lactic acid		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + KH <sub>2</sub> PO <sub>4</sub> + lactic acid		(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> + KH <sub>2</sub> PO <sub>4</sub> + sod citrate + lactic acid		Peptone water (control), pH 7.0
		* 2 cc	4 cc	* 2 cc	4 cc	* 2 cc	4 cc	
K G H	1	—	—	GG } PP }	GGG } ppp }	GGG } P }	GGG } P }	GGG } ppp }
"	2	—	—	"	"	"	"	"
"	3	—	—	"	"	"	"	"
"	4	—	—	"	"	"	"	"
"	5	—	—	"	"	"	"	"
"	6	—	—	"	"	"	"	"
"	7	—	—	"	"	"	"	"
"	8	—	—	"	"	"	"	"
"	9	—	—	"	"	"	"	"
"	10	—	—	"	"	"	"	"

\* Lactic acid (re-distilled) sp. g. 1.21, dilution 1 in 100 in sterile distilled water, pH adjusted to 7.4 in all using dilute potassium hydroxide solution.

Note — — = negative, no perceptible growth, G = slight growth, GG = good growth, GGG = profuse growth with pellicle formation, GGG } = profuse 'growth and pigment production

## DISCUSSION

The existing literature on the precise conditions of growth and pigment production of *Ps. pyocyanea* in synthetic media is not only meagre but also conflicting. Braun and Cohn-Bronner (1921) and Robinson (1932) cast doubt on the part played by magnesium in a medium containing carbon and nitrogen sources for cell metabolism. Braun and Cohn-Bronner (*loc cit*) reported that profuse growth was obtained in the absence of magnesium and hence it was not essential for growth, whereas Robinson (*loc cit*) could not get profuse growth in the absence of magnesium. No reference has been made by these workers to the part played by the individual ions constituting the medium nor to the variation of the hydrogen-ion concentration resulting from growth and pigmentary activity (Knight, 1938). Further, they did not recommend any simple synthetic medium well suited alike for profuse growth and pigment production. Robinson definitely mentioned that the amount of blue pyocyanin produced in synthetic media was small not comparable in any degree to that formed in peptone media.

In the present investigation, Table I shows that a medium containing ammonium chloride and sodium citrate is unsuitable for the growth of *Ps. pyocyanea*. The organism cannot break down these salts to meet its nutritional requirements, but the utilization of these salts is rendered possible if potassium dihydrogen phosphate is added to the medium. This observation throws light on the food requirements emphasizing the presence of phosphate as essential for growth and the easy dispensability of magnesium and sulphate ions from the medium. In any of the media shown in Table I, the organism could not synthesize pyocyanin at all though luxuriant growth was observed in media Nos 2, 4 and 5.

In Table II, sodium citrate is replaced by calcium lactate as carbon source. Such a change has induced luxuriant growth and the development of good amount of pyocyanin at the optimum pH 7.4 (medium No 3 in Table II). But when magnesium sulphate was also present (medium No 5 in Table II) conspicuous pigmentary activity developed, the period of incubation in both the cases being the same, viz 3 days. Thus, magnesium sulphate is proved to have some effect in stimulating pigment metabolism in the lactate medium. From the above experiments it is evident that calcium lactate can stimulate pigment production in synthetic media and that it is also a good source of carbon. Sodium citrate can, therefore, safely be replaced by calcium lactate.

From the observations recorded in Table III, it is evident that magnesium ions can be dispensed with from the synthetic media, for without this profuse growth and pigment production were both unaffected (medium No 2 in Table III), and, secondly, the sulphate ion was found to supply the necessary stimulus for pigmentary activity. In this case the growth as well as pigment production was similar to that obtaining in peptone media.

This prompts us to conclude that ammonium sulphate is a much better nitrogen source than ammonium chloride and also that sulphur may be an

essential nutritional requirement for cell metabolism. This is well supported by the fact that medium No 3 in Table III, wherein ammonium sulphate was replaced by ammonium sulphide, provided abundant growth with pellicle formation and pigment production.

The observations in Table IV show that lactic acid in proper dilution can alone induce pigmentary activity in synthetic media without the presence of calcium ions demonstrating that the former is an essential requirement for pigment production. Comparing media Nos 2 and 3 in Table IV it is seen that the presence of sodium citrate inhibits pigment production though it exerts no such effect on growth. Maximum growth and pigmentary activity are both observed at pH 7.4 with a final concentration of 1/900 lactic acid (sp g 121) and after incubation for 3 days.

It may be repeated that hydrogen-ion concentration exerts a profound influence specially in synthetic media (free from proteins) in controlling not only the growth activity but also all biochemical and metabolic activities of *Ps. pyocyanea*. Hence the pH factor should never be overlooked. The role of phosphate in synthetic media may be partly one of buffer action like that of peptone in complex peptone media. Thus, the problem of building up a synthetic medium for any organism resolves itself into two main factors, viz the pH factor and the nutritional factor for providing proper substrate.

The following two simple synthetic media were found suitable, one for profuse growth and the other for both profuse growth and pigmentary activity. The amount of pyocyanin produced in medium No 2 was found to stand a fair comparison with that in peptone media —

*Medium No 1* (for growth only)—

Ammonium sulphate	0.15 g
Potassium dihydrogen phosphate	0.1 g
Sodium citrate	0.2 g
Distilled water	100 ml
pH 7.0	

*Medium No 2* (for growth and pigment production)—

Ammonium sulphate	0.15 g
Potassium dihydrogen phosphate	0.1 g
Lactic acid (1/100)	80 ml
Distilled water	100 ml
pH 7.4	

# SUMMARY

1 The nutritional requirements of *Ps. pyocyanea* are studied employing ten strains.

2 Phosphate is found essential for growth and sulphur is found to improve pigment formation in synthetic media.

- 3 Lactic acid is essential for pigment production in the synthetic media studied
- 4 Magnesium is not essential either for growth or pigment production
- 5 Presence of proteins is not essential for pigmentary metabolism
- 6 Fairly large amounts of pyocyanin are formed in a protein-free synthetic medium otherwise suitably adjusted
- 7 A pH of 7.4 is found the optimum for growth and pigmentary activity
- 8 Two simple synthetic media are reported one for growth and the other for growth and pigment production

## ACKNOWLEDGMENTS

Our thanks are due to Dr N V Subrahmanyam of this department for his valuable help and also to Dr G Gopala Rao of the Andhra University for the interest shown in the investigation

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## A NEW METHOD OF ISOLATION OF VIBRIOS FROM CHOLERA STOOL

BY

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[Received for publication, March 31, 1942.]

WHILE searching for possible filter-passing forms of vibrios in cholera stools it was found that when a sample of cholera stool is put into an L<sub>3</sub> candle fitted into a wider test-tube containing peptone-water in such a position that the unglazed part of the candle is covered by the surrounding peptone-water and the whole is then incubated, vibrios from the stool grow through the candle into the peptone-water in 24 to 48 hours, and sometimes a pure culture is obtained by this procedure in 18 to 20 hours\* *Bact faecalis alkaligenes*, motile *coliform* organisms and late lactose-fermenters also grow through the candle but not so readily as the vibrios Dr de Monte of this School also noted at the same time that vibrios from a culture could grow through the candle into a surrounding culture medium If a small amount of the stool is mixed with peptone-water and partly aspirated through the candle into the surrounding peptone-water by vacuum action, growth of vibrios occurs earlier (18 to 20 hours)

Samples	Positive for vibrios.	
<i>First series of 57 samples of cholera stool —</i>	Per cent approximately	
(a) Stool mixed with peptone water, put into candle surrounded by peptone water	39	(68)
(b) Plating after peptone water enrichment (No candle)	28	(50)
(c) Direct bile salt agar plating	37	(65)

\* The above experiment does not demonstrate that vibrios have filter passing forms as no growth is seen to occur if the cholera stool is first filtered and then the filtrate is separately incubated

Samples	Positive for vibrios	
	Per cent approximately	
<i>Second series of 68 samples of cholera stool —</i>		
(a) Stool mixed with peptone water in candle placed in peptone water	42	(62)
(b) Direct bile salt agar plating	36	(53)
<i>Third series of 20 samples of cholera stool —</i>		
(a) Stool only in candle placed in peptone water (No peptone-water inside the candle )	14	(70)
(b) Direct bile salt agar plating	12	(60)

The above observations suggested that if some method of inhibiting the growth of *coliform* organisms could be included, a valuable method of isolating cholera vibrios could be evolved. Boric acid was found inhibitory to *coliform* organisms but not to the vibrios. A strength of 0.08 per cent was found to be suitable for the purpose. The acid was added to the peptone-water and the final pH was adjusted to 9.0. After 18 to 20 hours' incubation a pure growth of vibrios was found in most of the samples, although turbidity was not noticed in many.

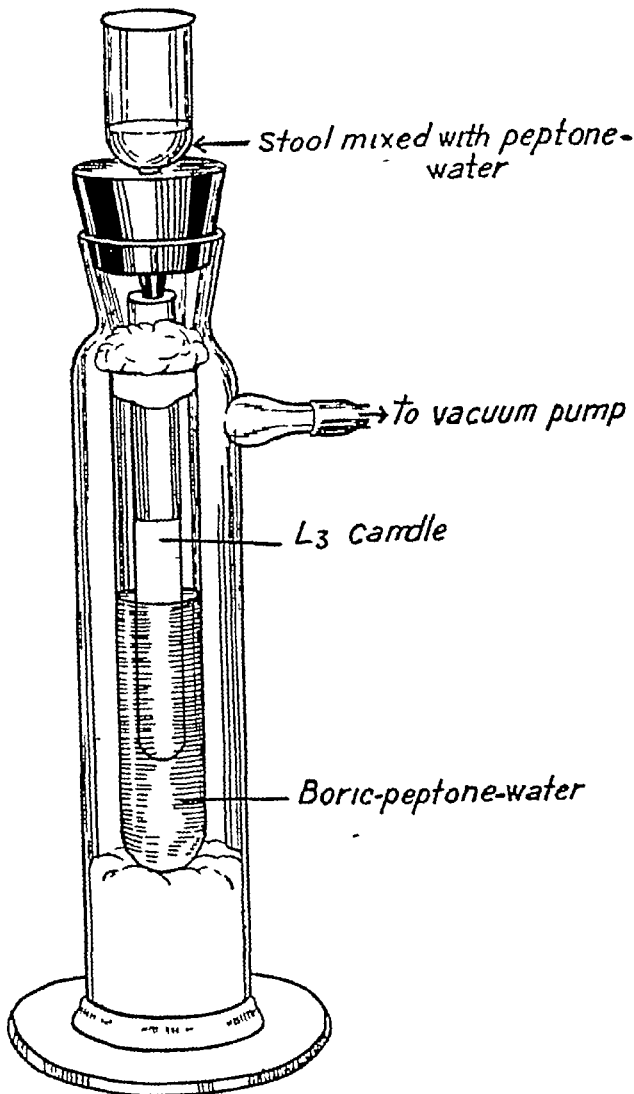
Vibrios were successfully isolated from only about 44 per cent of the samples on bile-salt agar in the declining period of an epidemic but from about 87 per cent of the same samples by the candle-boric-peptone-water method. It is expected that during the height of an epidemic the percentage of isolation would be even higher.

A comparison with other recent methods seemed desirable. Dr S. R. Pandit (unpublished) gives 81 per cent positive isolations during an epidemic and 92 per cent positive during the height of the same epidemic by plating on a modified Wilson and Reilly's solid medium.

A modified Wilson and Blair fluid medium (Read, 1939) kindly supplied by the Bengal Cholera Field Inquiry was used instead of boric-peptone-water outside the candle. The results are given below. The number of stools so far examined is small.

This new method is not differential for Inaba or Ogawa sub-types or El Tor and non-cholera vibrios.

Soil, natural waters, broth cultures of *Bact coli*, *Pr proteus*, *Bact faecalis*, *Ps pyocyanea*, *Bact typhosum*, *B subtilis* etc , were artificially mixed with a small



Isolation of vibrios by candle boric-peptone water method

number of vibrios and from such mixtures in 24 hours pure cultures of vibrios were usually obtained by the candle-boric-peptone-water method In 24 hours, impure

cultures were sometimes obtained and later the number of such impure cultures was higher. In all cases, however, vibrios were predominant.

*Forty-five samples of cholera stool examined during the declining period of an epidemic*

Methods	Number of samples from which vibrios were isolated	Percentage positive
(a) Direct bile salt agar plating	20	44
(b) Candle boric peptone water method	39	87

It is evident from the above results that the percentage of success is almost double by the new method when compared with direct bile-salt agar plating.

*Eleven samples of cholera stool —*

(a) Candle-boric-peptone-water method	11
(b) Candle in modified Wilson and Blair fluid medium	7

The Table shows the results of experiments with artificial mixtures. Most of these experiments have been repeated with almost the same results. Standard laboratory strains as well as freshly isolated strains have been used —

TABLE.

Mixtures	Direct plating on bile salt agar soon after mixing	Candle boric peptone water, 18 to 20 hours
Broth culture of <i>Bact coli</i> , 8 c c + one wire touch of Inaba sub type culture, either old laboratory culture or freshly isolated one	—	+++++++ Inaba pure
<i>Bact coli</i> 8 c c + Ogawa sub type	—	+++++++ Ogawa pure
<i>Bact coli</i> 8 c c + non cholera vibrio	+ or —	+++++++ Non cholera vibrios pure
<i>Bact coli</i> 8 c c + Inaba and Ogawa sub types and non cholera vibrios	+ or —	+++++++ Inaba, Ogawa, non cholera vibrios
<i>Pr proteus</i> (8 c c) + Inaba sub type culture (4 drops)	—	+++++++ Inaba pure

+ means a few vibrio colonies present    ++++++ means a large number of vibrio colonies    — means no vibrio colonies

TABLE—concl'd

Mixtures	Direct plating on bile salt agar soon after mixing	Candle boric peptone water, 18 to 20 hours
<i>Ps pyocyanea</i> (8 c.c.) + Inaba sub type culture (4 drops)	+	+++++++ Inaba pure
<i>Vibrio faecalis</i> (8 c.c.) + Inaba sub type culture (4 drops)	+	+++++++ Inaba pure
<i>Bact typhosum</i> (8 c.c.) + Inaba sub type culture (4 drops)	+	+++++++ Inaba pure
<i>B subtilis</i> (8 c.c.) + Inaba sub type culture (4 drops)	+	+++++++ Inaba pure
<i>Bact paratyph</i> A (8 c.c.) + Inaba sub type culture (4 drops)	—	+++++++ Inaba pure
<i>Bact carolinus</i> (8 c.c.) + Inaba sub type culture (4 drops)	—	+++++++ Inaba pure
<i>Bact coli</i> , <i>Bact faecalis</i> , <i>Pr proteus</i> , <i>Ps pyocyanea</i> , <i>Bact typhosum</i> and Inaba	—	+++++++ Inaba pure
Thick suspension of stool from non cholera cases 10 c.c. and Inaba sub-type culture one wire touch and rendered alkaline with N/10 NaOH	—	+++++++ Inaba pure, sometimes impure
Do + non cholera vibrios	—	+++++++ Non cholera vibrios pure, sometimes impure
A plateful of soil suspension + Inaba sub type culture, a few drops		+++++++ Inaba impure
Polluted water from a pool, 20 c.c. + 1 drop of Inaba sub-type culture		+++++++ Inaba pure, sometimes impure
Hooghly water 20 c.c. + 1 drop of Inaba sub type culture		+++++++ Inaba impure
Clean tank water + 1 drop of Inaba sub type culture		+++++++ Inaba pure
Drain water + 1 drop of Inaba sub type culture		+++ Inaba impure
+ means a few vibrio colonies present    ++++++ means a large number of vibrio colonies. — means no vibrio colonies		

It may be mentioned here that the method of peptone-water enrichment was often found less satisfactory than direct bile-salt agar plating for isolation of vibrios from cholera stools

*Vibrio faecalis* of Lehmann and Neumann (1896) as described by Nyberg (1935) was isolated in large numbers from several samples of cholera stool by the new

method The organism was found non-pathogenic to guinea-pigs and was biochemically and serologically distinct though morphologically it was often indistinguishable from *V. cholera*

The advantages of the new method are —

- (i) Higher percentage of isolation than by other methods
- (ii) Large numbers of colonies are available for slide-agglutination test and typing
- (iii) A pure culture is obtained on many occasions

To ensure success candles should be tested before sterilization for patent porosity and leakage by forcing air under pressure of 15 lb to 20 lb above the atmospheric pressure, while the candles are immersed in water. If no air passes through, blockage is indicated and if large bubbles come out there is leakage.

#### SUMMARY

A new method of isolation of vibrios from cholera stool is described.

Cholera stool mixed with a little peptone-water is first put into an L<sub>3</sub> porcelain candle and then the candle is placed in boric(0.08 per cent)-peptone-water at pH 9.0. By suction, a little of the stool mixture is drawn through the candle and the whole is next incubated for 24 to 48 hours.

Vibrios grow readily through the candle into boric-peptone-water causing a faint turbidity, and an almost pure culture is often obtained on plating the turbid fluid on bile-salt agar. Boric acid inhibits the *coliform* organisms but not the vibrios.

Compared with standard methods of isolation of vibrios, this new method gives a higher percentage of isolation.

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## A SIMPLE PROCEDURE FOR ESTIMATING NICOTINIC ACID IN BIOLOGICAL MATERIALS USING THE-CYANOGEN BROMIDE-ANILINE REAGENT

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[Received for publication, March 31, 1942]

NUMEROUS modifications in the cyanogen bromide method of estimating nicotinic acid in biological materials, first described by the author (Swaminathan, 1938), have been suggested by various workers. In a recent publication (Swaminathan, 1941b) evidence has been produced to show that in general these modifications do not lead to more satisfactory results than the technique followed in the Nutrition Research Laboratories. It was also shown that aniline (the aromatic amine used by the author throughout) gives as intense a colour as various aromatic amines used by other investigators, viz  $\beta$ -naphthylamine, metol and *p*-aminoacetophenone (von Euler, Schlenk, Heiwinkel and Hogberg, 1938, Bandier, 1939, Bandier and Hald, 1939, Harris and Raymond, 1939, Kodicek, 1940). Further, it was pointed out that certain side reactions of the aromatic amines, which have been reported to occur under certain conditions by various workers (Bandier and Hald, *loc cit*, Harris and Raymond, *loc cit*, Kodicek, *loc cit*, Melnick and Field 1940a, b), do not take place with aniline, when the reaction is performed in an aqueous medium at pH 7. To overcome difficulties arising from side reactions, Bandier and Hald (*loc cit*) recommended the addition of cyanogen bromide instead of metol to the 'blank', while Melnick and Field (1940a) found it necessary to omit both cyanogen bromide and aniline from the 'blank' and preferred the use of a 'dilution blank'. These innovations have not proved altogether successful, for Waisman and Elvehjem (1941), using the method of Melnick and Field (1940a, b), reported very high values, 10.5 mg and 10.7 mg per 100 g, for polished rice and maize respectively. Such high values, which do not conform with those given by other workers, are presumably due to the side reactions of the aromatic amine which invariably occur in aqueous alcoholic solutions. Bandier (*loc cit*) found that reliable values for the nicotinic acid content of urine were obtained



only when hydrolysis with alkali was followed, acid hydrolysis yielded values two to three times higher, presumably due to interference from other unknown substances. Such discrepancies are never encountered when the estimations are carried out in aqueous medium at pH 7 using aqueous aniline.

Recently, Giri and Naganna (1941) and Dann and Handler (1941) have described adsorption methods for the separation of nicotinic acid from other interfering substances. The use of adsorption procedures in tests for vitamins of the B group is not new. They have been extensively used for the separation of vitamin B<sub>1</sub>, riboflavin and vitamin B<sub>6</sub> from interfering substances and are usually resorted to when a direct estimation is impossible owing to the presence of interfering substances. Further quantitative adsorption and elution of substances are, however, not always possible. This statement is substantiated by the results reported by Giri and Naganna (*loc cit*) who found that the recovery of nicotinic acid added to foods ranged from 40 to 108 per cent, with the result that they obtained a low value of 0.2 mg and 0.4 mg per 100 g in the case of maize instead of values ranging from 1 mg to 1.4 mg which are those usually obtained.

The method previously described by the author (Swaminathan, 1938) has been used in this Laboratory during the past 3 years and has proved reliable and accurate for the estimation of nicotinic acid in foods and urine (Swaminathan, 1939, 1940, 1941a, Aykroyd and Swaminathan, 1940, Shourie and Swaminathan, 1940). The reliability of the method has recently been confirmed by Saha (1941) and Ghosh (1941) working with fish and urine respectively. It has been found possible to do 2 to 4 estimations in a day. In the present communication a simpler procedure is described which yields results corresponding well with those given by the previous method. By following this method, a single worker can estimate the nicotinic acid content of 6 test substances in 3 hours and 12 in one day.

### EXPERIMENTAL

The reagents required are the same as those described before (Swaminathan, 1938, 1939).

The method consists of the following steps —

- (1) Extraction of the nicotinic acid and its derivatives present in foods and animal tissues using 2 N HCl followed by hydrolysis of the nicotinamide present in the extracts to nicotinic acid.
- (2) Removal of protein derivatives and colouring matter by treatment successively with barium acetate at pH 6 and zinc hydroxide at pH 9 and the excess of Ba as sulphate.
- (3) Colorimetric estimation of nicotinic acid at pH 7 in an aqueous medium using aqueous aniline.

### *Procedure*

(1) *Extraction and hydrolysis* — A weighed amount of the finely powdered food material (1 g to 25 g), containing from 50  $\mu$ g to 400  $\mu$ g nicotinic acid, was placed

in a conical flask Eighty ml of water were then added and the mixture heated in a water-bath maintained at 70°C to 80°C for 10 minutes, with constant stirring Twenty ml of concentrated HCl (36 per cent) were then added and the heating continued for another 10 minutes The mixture was then cooled under the tap and centrifuged Fifty ml of the clear extract were transferred to a beaker (100 ml capacity) and heated in a boiling water-bath for 40 minutes to hydrolyse the nicotinamide present to nicotinic acid

(2) *Removal of protein derivatives and colouring matter*—After hydrolysis, the extract was cooled and neutralized to pH 5 to 6 by the careful addition of 50 per cent sodium hydroxide Five ml of N barium acetate solution were then added and the total volume of the mixture was adjusted to 60 ml by the addition of water The precipitate formed was removed on the centrifuge To the clear centrifugate, 1 ml of 20 per cent zinc sulphate solution was added Zinc hydroxide was precipitated by the careful addition of N NaOH (15 ml to 2 ml. is required) with phenolphthalein as internal indicator, so that the mixture was very faintly pink in colour and the pH about 9.5 Excess of sodium hydroxide should be avoided as it may dissolve part of the precipitate The precipitate was removed on the centrifuge, the excess of barium present precipitated by the addition of 1 ml of 5 N H<sub>2</sub>SO<sub>4</sub>, and the precipitate removed on the centrifuge The clear extract was adjusted to pH 7 by the addition of 5 N NaOH (about 0.8 ml to 1.2 ml will be required) and filtered Thirteen ml of the final clear solution, representing 10 ml of the original extract and one-tenth of the weight of the material taken for estimation, were used for the colorimetric estimation The solutions were in general almost colourless but in certain cases were coloured light yellow which was allowed for by a 'blank' estimation

(3) *Colorimetric estimation of nicotinic acid*—Aliquots of the extract (usually 13 ml = one-tenth of the weight of the material taken) were measured out in a series of 25-ml measuring flasks Standard nicotinic acid (20 µg) was taken in another flask and the volume of the standard diluted to 13 ml by the addition of distilled water Half a ml of 50 per cent sodium acetate solution (adjusted to pH 7) was then added to each flask One ml of 2 per cent aqueous aniline was added to all the flasks, followed by 6 ml of cyanogen bromide solution The contents of the flasks were shaken and allowed to stand for one minute The volume was then made up to 25 ml by the addition of aqueous aniline, the contents of the flasks were well mixed and allowed to stand for one minute The colours were compared immediately (within 10 minutes) A 'blank' estimation was carried out in the above manner for all the unknown solutions, with the exception that distilled water was added instead of CnBr, and the values so obtained for the 'blank' were subtracted in the usual manner from the test values to obtain the true values When 25 g of fresh vegetables low in nicotinic acid were taken for analysis, the volume of the extract was usually 115 ml, though only 100 ml of 2 N HCl were used for extraction In such cases the volume of the extract was taken as 115 ml for calculation

The recovery of added nicotinic acid ranged from 95 to 100 per cent. The results obtained with 16 foodstuffs containing varying amounts of nicotinic acid (0.2 mg to 47.4 mg per 100 g) are given in the Table. Values obtained for the same foodstuffs by following the procedure described before (Swaminathan, 1938) are also given for comparison —

TABLE

*The nicotinic acid content of foodstuffs (mg per 100 g)*

Name of foodstuff	New procedure as described in the text	Old procedure (Swaminathan, 1938)
<i>Cereals —</i>		
Cambu ( <i>Pennisetum typhoides</i> )	3.2	2.8
Cholam ( <i>Sorghum vulgare</i> )	1.8	1.7
Italian millet ( <i>Setaria italica</i> )	0.7	0.8
Maize, whole, yellow	1.4	1.3
Ragi ( <i>Eleusine coracana</i> )	1.2	1.3
Rice, raw, husked	4.1	3.5
Rice, raw, milled	1.6	1.3
Rice, parboiled, milled	4.0	3.5
Wheat, whole	4.6	4.3
<i>Pulses —</i>		
Black gram ( <i>Phascolus mungo</i> )	1.6	1.7
Green gram ( <i>Phaseolus radiatus</i> )	1.7	1.9
Lentil ( <i>Lens esculenta</i> )	1.5	1.8
Red gram ( <i>Cajanus indicus</i> )	1.9	2.1
<i>Vegetables —</i>		
Cabbage	0.3	0.3
Carrots	0.2	0.2
<i>Miscellaneous —</i>		
Skimmed milk powder	1.0	1.1
Yeast, dried, brewers	44.5	48.3
Yeast, dried (torula strain grown on molasses-salts medium)	19.2	20.1

## DISCUSSION

The above method is simpler than the one previously described by the author and those described by Giri and Naganna (*loc cit*) and Dann and Handler (*loc cit*). It is comparable in simplicity with the method elaborated by Melnick and Field (*loc cit*). It has, however, an important advantage over the latter method in that the complications caused by the use of aniline in an aqueous alcoholic medium do not occur when the reaction is carried out in an aqueous medium at pH 7, the aniline 'blank' and the dilution 'blank' always giving the same value. The added nicotinic acid is quantitatively recovered.

## SUMMARY

1. A simple procedure is described for the estimation of nicotinic acid in food-stuffs using the cyanogen bromide-aniline reagent.
2. Nicotinic acid and its derivatives were extracted with 2 N HCl and part of the extract heated in a boiling water-bath for 40 minutes to hydrolyse the nicotinamide present to nicotinic acid. Protein derivatives and colouring matter present were removed by treatment with barium acetate at pH 6 and zinc hydroxide at pH 9.5 and the excess of barium as sulphate.
3. Nicotinic acid present in aliquots of the extract was estimated colorimetrically by treatment with cyanogen bromide and aqueous aniline at pH 7.

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## NOTE ON THE VITAMIN B<sub>1</sub>, RIBOFLAVIN AND NICOTINIC ACID CONTENT OF DRIED YEAST

BY

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[Received for publication, March 31, 1942]

YEAST contains all the B vitamins and since almost the earliest days of vitamin research has been used as a source of the complex in experimental diets, and also in clinical medicine. The fractionation of yeast has proved a most fruitful line of research on the vitamin B complex and has greatly helped in the isolation and identification of the various components which the complex includes. While in general yeast is rich in 'vitamin B', it is not a constant and unvarying source of the different members of the group. There are numerous strains of yeast and these may vary in their capacity to synthesize the different factors, or extract them from the growth medium. Again, the nature of the medium in which the yeast cells are propagated influences the composition of yeast. It is to be expected that yeast grown in a medium rich in B vitamins will contain more of these vitamins than yeast grown in poorer media.

Quinn, Whalen and Hartley (1930) found by the rat-growth test that the concentration of vitamin B<sub>1</sub> in baker's yeast was lower than in brewer's yeast, while that of vitamin B<sub>2</sub> was approximately similar in both kinds of yeast. Copping and Roscoe (1937) have made similar observations. More recently, Pavcek, Peterson and Elvehjem (1937) have studied the effect of variation in the composition of the medium in which yeast was grown, on the vitamin B<sub>1</sub> content of yeast. They found that baker's yeast (*Saccharomyces cerevisia*) contained 30  $\mu\text{g/g}$  of vitamin B<sub>1</sub> dry matter when grown in a grain-wort medium but only 15  $\mu\text{g}$  and 9  $\mu\text{g}$  when grown in molasses-salt and glucose-salt media respectively. Peterson and Elvehjem (1939) have also reported a similar variation in the pantothenic acid content of the same yeast samples.

Dried yeast has been widely used in Indian hospitals in the treatment of deficiency states. Previous to the war, a good deal of imported yeast was used, but as the war proceeded it became clear that the supply of imported material might fail. The investigation of alternative sources thus assumed importance. In the present investigation, three kinds of yeast were studied, their vitamin B<sub>1</sub>, nicotinic acid and riboflavin content being determined. The amount of these food factors present provides a reasonably satisfactory criterion of the nutritive value of yeast and its suitability for therapeutic purposes. The kinds of dried yeast tested were as follows —

(a) *Brewer's yeast* —Dried yeast for medicinal purposes has been for some years prepared and sold by breweries in India. The number of breweries is, however, small, and the supply appears to be unequal to the demand. Four samples were tested, 3 from an Indian brewery and one from a brewery in Ceylon.

(b) *Dried 'food yeast' grown on molasses* —Yeast can be produced specifically for human consumption by suitable industrial methods. The principles underlying the latter are the use of some cheap source of carbohydrate food material, such as sugar cane or beet molasses, and strong aeration to suppress the formation of alcohol. Molasses is available in abundance in India and a proportion of the surplus might be devoted to yeast production to supply both Indian and foreign markets. It has been found that one particular strain of yeast, *Torula utilis*, is particularly suitable for use in the manufacture of food yeast, since it gives large yields when grown in a molasses solution and is palatable when dried.

Experiments have been in progress in the Laboratories on the production of food yeast. The yeast was propagated in a 1-per cent solution of molasses to which ammonium phosphate and super-phosphate, 2 g to 5 g respectively per 100 g of molasses, were added. The mixture was strongly aerated during fermentation. Yields of dried yeast equivalent to 20 to 25 per cent of the weight of molasses were obtained, though not consistently.

Two strains of yeast were propagated in this way. One of these, kindly supplied by the Department of Biochemistry, Indian Institute of Science, Bangalore, was described as being 'closely related to *Torula utilis*'. The other was a strain of *Torula utilis* obtained from the Chemical Research Laboratory, Teddington, England. Eight samples of dried torula yeast were tested. All the samples grown on molasses were prepared by Mr A R Sundararajan.

(c) *Distillery yeast* —This is produced by drying the 'sludge' run off from distillery vats after fermentation. No aeration is applied in the manufacture of alcohol. It is well known that in general the greater the production of alcohol during fermentation, the lower the yield of yeast, and vice versa. Owing to the shortage of yeast in India, it was proposed to use dried distillery yeast for medicinal purposes. Hence it was desirable to obtain data about its vitamin content. Three samples were tested.

A sample of Marmite was also tested for purposes of comparison.

*Methods*—The methods used for determining the vitamin B<sub>1</sub>, riboflavin and nicotinic acid content of yeast were those previously described by Swaminathan (1938, 1942a, b) The results are shown in the Table —

TABLE

*Vitamin B<sub>1</sub>, riboflavin and nicotinic acid content of dried yeast*

Sample number	Variety	Vitamin B <sub>1</sub> , μg /g	Riboflavin, μg /g	Nicotinic acid, μg /g
1	Yeast, dried, brewer's	42.1	52.3	464
2	Do	34.0	55.4	430
3	Do	21.0	44.6	400
4	Do	33.6	48.5	412
5	Yeast, dried torula, grown in synthetic medium containing (1)	26.1	64.8	195
6	Do (1)	25.4	55.6	165
7	Do (1)	24.1	71.5	180
8	Do (1)	22.8	84.7	261
9	Do (1)	33.3	62.0	200
10	Do (2)	33.3	68.0	190
11	Do (2)	41.2	55.0	200
12	Do (2)	28.0	74.0	337
13	Yeast, dried, distillery	12.9	32.5	90
14	Do	10.4	23.2	65
15	Do	11.2	22.4	63
16	Marmite	12.5	89.2	665

## RESULTS OF TESTS

*Vitamin B<sub>1</sub> content*—The vitamin B<sub>1</sub> content of the four samples of brewer's yeast ranged from 21 μg /g to 42 μg /g of dried yeast. Torula yeast gave values of 24 μg /g to 41 μg /g. Dried distillery yeast was poorer, containing only 10 μg /g to 12 μg /g. Using the 'bradycardia' method, Baker and Wright (1935) reported values of 18 μg /g and 69 μg /g respectively for two samples of dried brewer's yeast, while Leong (1940) has given a value of 22.5 μg /g for dried yeast (proprietary brand). Values ranging from 24 μg /g to 490 μg /g have been obtained by Hennessy and Cerecedo (1939) who employed the thiochrome method. The value



obtained for Marmite, 12.5  $\mu\text{g/g}$ , was lower than that recorded by Birch and Harris (1934) using the bradycardia method (25  $\mu\text{g/g}$ ). More recently, Leong (*loc cit*), using the bradycardia method, has reported the low value of 6  $\mu\text{g/g}$ . The age of the sample tested in the Laboratories was not known and it is possible that loss in vitamin B<sub>1</sub> content had occurred on storage.

*Riboflavin*—The riboflavin content of 4 samples of brewer's yeast ranged from 44  $\mu\text{g/g}$  to 55  $\mu\text{g/g}$ . The torula yeast samples gave higher values, varying from 55  $\mu\text{g/g}$  to 84  $\mu\text{g/g}$ . Distillery yeast was poorer, containing 22  $\mu\text{g/g}$  to 32  $\mu\text{g/g}$ .

The protein ( $\text{N} \times 6.25$ ) content of brewer's and torula yeast ranged from 35 to 50 per cent, while that of distillery yeast was in the neighbourhood of 30 per cent.

*Nicotinic acid*—The nicotinic acid content of the brewer's yeasts ranged from 400  $\mu\text{g/g}$  to 460  $\mu\text{g/g}$ . The torula samples contained 200  $\mu\text{g/g}$  to 330  $\mu\text{g/g}$ , while distillery yeast gave lower values of 60  $\mu\text{g/g}$  to 90  $\mu\text{g/g}$ . The sample of Marmite was richer in nicotinic acid than any of the dried yeast samples.

According to an English report (private communication) the values for the vitamin content of food yeast are approximately as follows: vitamin B<sub>1</sub>, 24  $\mu\text{g/g}$ ; riboflavin, 60  $\mu\text{g/g}$ ; and nicotinic acid, 285  $\mu\text{g/g}$  to 455  $\mu\text{g/g}$ . The corresponding values for brewer's yeast were 18  $\mu\text{g/g}$  to 138  $\mu\text{g/g}$ , 18  $\mu\text{g/g}$  to 124  $\mu\text{g/g}$  and 300  $\mu\text{g/g}$  to 400  $\mu\text{g/g}$  respectively. The protein content of both kinds of yeast given was about 50 per cent. Values of the same order were obtained for the samples of brewer's and torula yeasts tested in this investigation.

## DISCUSSION

Dried brewer's yeast prepared in India and Ceylon was found to have a vitamin content conforming reasonably well with that reported by other workers for brewer's yeast. It can be recommended for medicinal purposes. Distillery yeast is less suitable, being lower in all 3 factors, so that a considerably larger dose would have to be given to provide the same amount of vitamins. Clinicians used to prescribing imported dried yeast or locally produced brewer's yeast would find distillery yeast less effective in the same doses. It can, of course, be said that it has some value.

Dried torula yeast grown on molasses solution gave satisfactory values, particularly as regards riboflavin. Torula yeast is much more palatable than brewer's yeast. Recent work (Aykroyd and Verma, 1942) has shown that riboflavin deficiency is very common in India. The production of dried torula yeast on a large scale would be of great value, provided it could be sold at a cheap rate. A surplus of molasses is available, and a number of schemes for its utilization have been considered within recent years. The conversion of a relatively valueless material into food rich in important vitamins greatly needed to make good the defects of Indian diets, is obviously a sensible procedure.

## SUMMARY

1 The vitamin B<sub>1</sub> riboflavin and nicotinic acid content of 15 samples of dried yeast, including 4 samples of brewer's yeast, 8 samples of torula yeast grown on a molasses-salts medium and 3 samples of distillery yeast has been determined using chemical methods

2 The torula samples were found to compare favourably in their vitamin content with brewer's yeast. The distillery yeast samples were found to be poorer sources

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## THE EFFECT OF WASHING AND COOKING ON THE VITAMIN B<sub>1</sub> CONTENT OF RAW AND PARBOILED MILLED RICE

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[Received for publication, March 31, 1942]

THE fact that milled parboiled rice has a higher vitamin B<sub>1</sub> content than raw milled rice is now well known. Its bearing on the epidemiology of beriberi in India has been demonstrated by Aykroyd, Krishnan, Passmore and Sundararajan (1940), who showed that beriberi is rare among populations consuming machine-milled parboiled rice.

It was observed by Aykroyd and Swaminathan (1940) that the nicotinic acid present in rice behaves in the same way as vitamin B<sub>1</sub> during the process of parboiling, washing and cooking. In a subsequent investigation the author (Swaminathan, 1941) studied in detail the effect of these processes on nicotinic acid in rice, assuming that data about changes in nicotinic acid content would be applicable to vitamin B<sub>1</sub>. As a result of this investigation the following conclusion was reached: 'In connection with the beriberi problem, parboiled milled rice has two advantages over raw milled rice: (a) it has a higher vitamin B<sub>1</sub> content and (b) the vitamin B<sub>1</sub> present in the former is more difficult to wash out. Both are of importance in producing the almost complete immunity of the parboiled rice-eater to beriberi.'

This conclusion, based on an analogy between the behaviour of vitamin B<sub>1</sub> and nicotinic acid, is of considerable practical and public health importance. It was felt desirable to confirm it by an investigation of vitamin B<sub>1</sub> itself under similar conditions, making use of an improved thiochrome method for estimating vitamin B<sub>1</sub> recently worked out in the Laboratories (Swaminathan, 1942).

Previous work on the effect of washing on the vitamin B<sub>1</sub> content of rice may be briefly referred to. McCarrison and Norris (1924) showed by biological tests on pigeons that excessive washing of rice, whether raw or parboiled, greatly reduced

the vitamin B<sub>1</sub> content Subsequently, van Veen (1933) in Java, using biological methods, produced quantitative evidence to show that excessive washing of raw rice, whether husked undermilled or milled, removed a considerable portion of the vitamin B<sub>1</sub> originally present Yang (1938) reported that raw rice, husked or milled, lost a greater part of its vitamin B<sub>1</sub> during the process of washing while the loss from parboiled rice was much smaller Aykroyd *et al* (*loc cit*) found that the washing and cooking of raw rice resulted in a loss of about 50 per cent of the vitamin B<sub>1</sub> present when the cooking water was discarded

### EXPERIMENTAL

Samples of raw and parboiled milled rice were purchased from the local market These were of the type commonly consumed in South India The vitamin B<sub>1</sub> content of the original samples was determined Then the rices were washed and cooked according to practices commonly followed in Indian homes Rice is usually washed several times to remove dirt and other adhering foreign matter before being cooked The method of cooking varies in different communities in that the cooking water ('conjee') is discarded by some, while others consume it The vitamin B<sub>1</sub> estimations were carried out on the wash-water cooked rice and 'conjee' The vitamin content of washed rice was calculated by difference The loss of vitamin B<sub>1</sub> from raw and parboiled rice during the above processes was compared

The method followed in washing and cooking of rice was the same as that described in the previous paper (Swammathan, 1941)

*Estimation of vitamin B<sub>1</sub>*—In carrying out tests on the water used for washing and cooking the following procedure was adopted —

*Wash-water*—Five ml of a N solution of lead acetate were added to 100 ml of the wash-water and the precipitate removed on the centrifuge Excess of lead was removed as sulphate The clear solution so obtained was used in aliquots of 5 ml to 20 ml for the estimation of the vitamin present

*'Conjee'*—The 'conjee' was clarified by zinc hydroxide, as treatment with lead acetate did not yield clear solutions

Ten ml of 4.5 per cent zinc sulphate solution were added to 100 ml of 'conjee' Two ml of N NaOH were then added slowly with constant stirring to precipitate zinc hydroxide The precipitate was removed on the centrifuge and the clear filtrate was acidified by the addition of 1 ml of 10 N H<sub>2</sub>SO<sub>4</sub> Ten ml to 20 ml of the clear solution were used for the estimation of the vitamin by the thiochrome reaction

The recovery of added vitamin ranged from 50 to 70 per cent and all the values were corrected by applying the corresponding recovery values

### RESULTS

The results obtained are shown in Tables I to III —

TABLE I

*The effect of washing on the vitamin B<sub>1</sub> content of raw and parboiled milled rice*

Sample number	Vitamin B <sub>1</sub> content, $\mu\text{g}/\text{g}$	VITAMIN B <sub>1</sub> LOST IN WASH WATER ( $\mu\text{g}$ PER g OF ORIGINAL RICE)				Percentage loss in washings	Washed rice vitamin B <sub>1</sub> , $\mu\text{g}/\text{g}$ (by difference)
		1st washing	2nd washing	3rd washing	Total		
Raw milled 1 2 3 4 5	1.0	0.5	0.1	0.05	0.05	0.5	0.35
	1.2	0.1	0.1	0.1	0.00	50	0.00
	1.0	0.5	0.1	0.03	0.03	0.3	0.37
	0.9	0.1	0.1	0.04	0.54	60	0.17
	0.7	0.3	0.08	0.03	0.11	60	0.20
AVERAGE	1.0				0.00	60	0.1
Parboiled milled 1 2 3 4 5	1.0	0.1	0.00	0.01	0.20	10	1.70
	2.1	0.1	0.05	0.02	0.17	7	2.23
	1.0	0.1	0.03	0.03	0.16	8	1.74
	2.2	0.1	0.02	0.02	0.14	0	2.00
	2.0	0.1	0.02	0.02	0.14	7	1.80
AVERAGE	2.1				0.2	8	1.9

TABLE II  
*The effect of washing with varying amounts of water on the vitamin B<sub>1</sub> content of raw and parboiled milled rice*

Sample number	Vitamin B <sub>1</sub> content, $\mu\text{g/g}$	Quantity of water used	VITAMIN B <sub>1</sub> LOST IN WASH-WATER ( $\mu\text{g}$ PER g OF ORIGINAL RICE)			Percentage loss in washings	Washed rice vitamin B <sub>1</sub> , $\mu\text{g/g}$ (by difference)
			1st washing	2nd washing	3rd washing	Total	
Raw milled $\left\{ \begin{array}{l} 2 \\ 2 \end{array} \right.$	1.2	Equal weight	0.4	0.1	0.1	0.6	0.60
	1.2	4 times weight	0.4	0.1	0.1	0.6	0.60
Parboiled milled $\left\{ \begin{array}{l} 2 \\ 2 \end{array} \right.$	2.4	Equal weight	0.1	0.05	0.02	0.17	2.23
	2.4	4 times weight	0.1	0.1	0.04	0.24	2.16

TABLE III

*The effect of washing and cooking on the vitamin B<sub>1</sub> content of raw and parboiled milled rice*

Sample number	Vitamin B <sub>1</sub> content, μg/g	Wash water (μg per g of original rice)	Percentage loss in washings	Washed rice, μg/g (by difference)	COOKED RICE		Percentage of vitamin B <sub>1</sub> present in washed rice removed on cooking
					(μg per g of original rice)		
Raw milled <div><div>1</div><div>2</div></div>	1.0	0.6	60	0.4	0.2	0.1	25
	1.2	0.6	50	0.6	0.3	0.2	33
AVERAGE	1.1		55		0.25		30
Parboiled milled <div><div>1</div><div>2</div></div>	1.9	0.2	10	1.7	1.2	0.4	25
	2.4	0.2	8	2.2	1.6	0.5	25
AVERAGE	2.2		9		1.4		25



*Effect of washing*—Table I shows that the average loss of vitamin B<sub>1</sub> from raw rice on washing was 60 per cent, whereas with parboiled milled rice the corresponding figure was only 10. The first washing removed most of the vitamin, the second and third washings containing but little. It will be seen from Table II that even washing with large amounts of cold water failed to remove more vitamin from parboiled rice samples. Washed parboiled milled rice contained 4 to 5 times as much vitamin B<sub>1</sub> as washed raw milled rice, the average values obtained being 1.9  $\mu\text{g}$  and 0.4  $\mu\text{g}$  per g respectively.

*Effect of cooking*—Table II shows a further 25 per cent of the vitamin contained in washed raw and parboiled rice is dissolved into the cooking water. Since the vitamin B<sub>1</sub> content of washed parboiled rice is about 4 times that of washed raw rice, the 'conjee' obtained from parboiled rice is correspondingly 3 to 4 times as rich as 'conjee' from raw rice. It is to be noted that cooked parboiled rice, even after the losses taking place on washing and cooking, still contained 1.4  $\mu\text{g}$ /g, whereas the cooked raw milled rice was a poor source of the vitamin, containing only 0.25  $\mu\text{g}$ /g.

*Vitamin B<sub>1</sub>/calorie value of washed and cooked raw and parboiled rice*—The vitamin B<sub>1</sub>/calorie value of untreated, washed and cooked rices was calculated on the assumption that the average calorie content of rice is 350 per cent. Ranganathan, Sundararajan and Swaminathan (1937) have reported calorie values ranging from 300 to 350 for untreated and cooked rice samples. The results are shown in Table IV—

TABLE IV

*The average vitamin B<sub>1</sub>/calorie ratio of washed and cooked raw and parboiled rices*

Rice	Vitamin B <sub>1</sub> , $\mu\text{g}$ /g	Vitamin B <sub>1</sub> /calorie ratio
Raw milled (original sample)	1.0	0.20
Raw milled, washed	0.4	0.11
Raw milled, washed and cooked	0.25	0.08
Parboiled milled (original sample)	2.1	0.60
Parboiled milled, washed	1.9	0.54
Parboiled milled, washed and cooked	1.4	0.40

## DISCUSSION

Williams and Spies (1938) calculated the vitamin B<sub>1</sub>/calorie ratio of a long series of diets and showed that diets giving a value less than 0.25 were likely to be associated with beriberi. Aykroyd *et al* (*loc cit*) pointed out that the minimum vitamin B<sub>1</sub> content of rice in typical poor Indian rice diets necessary to make the vitamin B<sub>1</sub>/calorie ratio greater than 0.26, i.e. to raise it above the beriberi-producing level according to the Williams and Spies criterion, ranged from 0.69  $\mu\text{g/g}$  to 0.84  $\mu\text{g/g}$ . It will be seen from Table IV that untreated raw rice samples contain a little more than the required amount of vitamin B<sub>1</sub>, whereas washed and cooked raw rice, containing much less, is definitely 'beriberi producing' according to this criterion. On the other hand, parboiled milled rice after washing and cooking (even when the 'conjee' is discarded) supplies more vitamin B<sub>1</sub> than the minimum required to prevent beriberi.

The present investigation further clarifies the observation made by Aykroyd *et al* (*loc cit*) that beriberi as a serious public health problem occurs only in places where raw milled rice is consumed by the poor, e.g. the Northern Circars district of the Madras Presidency, Malaya, China, Java and Japan, and is rare when parboiled milled rice is the chief staple, as in most parts of South India. It also confirms the validity of the previous conclusion that the nicotinic acid and vitamin B<sub>1</sub> in rice are affected in the same way by the various processes to which the grain may be subjected before consumption. Actually the percentage losses of the two food factors are almost precisely the same. The findings are essentially similar to those of Yang (*loc cit*), whose paper was not available in Coonoor when the previous investigation on nicotinic acid in the rice grain was carried out.

Platt (1939) has shown that the washing of raw rice is an important factor in the causation of beriberi in China. The consumption of washed raw milled rice may lead to beriberi, whereas unwashed raw milled rice may contain enough vitamin B<sub>1</sub> to prevent the disease. This again is fully in line with the facts presented above.

The investigation confirms the original hypothesis of Aykroyd (1932) that 'when rice is parboiled the endosperm absorbs vitamin B<sub>1</sub> at the expense of the germ and pericarp and milling does not remove the vitamin'. In raw rice most of the vitamin is located at the surface of the grain and is readily washed away. In parboiled rice it is located more deeply and is less easily dissolved out by the water used in washing.

## SUMMARY

1 The effect of washing and cooking on the vitamin B<sub>1</sub> content of commercial samples of raw and parboiled rice has been investigated.

2 Raw milled rice loses the greater part of its vitamin B<sub>1</sub> (60 per cent) during the process of washing while parboiled milled rice, in spite of its higher vitamin B<sub>1</sub> content, loses much less (8 per cent). Washed parboiled rice contains on the average 4 times as much vitamin B<sub>1</sub> as washed raw rice.

3 Another 25 per cent of the vitamin contained in washed raw and parboiled rice is dissolved out with the cooking water. Cooked parboiled rice, even when the cooking water is discarded, contains enough vitamin B<sub>1</sub> to prevent the occurrence of beriberi according to the Williams and Spies criterion

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## STUDIES IN HUMAN NUTRITION

### Part V

#### THE BONES OF SMALL FISH AS A SOURCE OF NUTRITIONALLY AVAILABLE CALCIUM AND PHOSPHORUS

BY

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*An Inquiry under the Indian Research Fund Association*

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[Received for publication, February 19, 1942]

PREVIOUS communications from this Laboratory (Basu Basak and Rai Sircar, 1939, Basu *et al* 1941, 1942) on the results of metabolic studies on human beings have revealed that the lack of adequate amounts of calcium constitutes one of the greatest defects of typical Indian dietaries. These communications have also described our attempts to discover suitable supplements to meet this deficiency. Ten ounces of milk or the amount of lime ingested in the process of chewing four betel-leaves, prepared with lime, were shown to make good the calcium deficiency of typical Indian dietaries. The present paper deals with the availability of calcium from another cheap source.

Fish forms an important ingredient in the rice-eaters' diet in Bengal and certain other parts of India. The quality of proteins of different varieties of fish and also the vitamin A and D contents of the liver and body-oils of various species of fish have been the subject of a series of investigations from this

Laboratory (Basu and De, 1938*a, b, c*, Basu and Gupta, 1939, Basu *et al*, 1940*a, b*) When comparatively bigger fish are incorporated in the diet, only the fleshy portion is taken, the bones being usually discarded. But when very small fish are taken the whole fish including the bones, which are generally quite soft, are masticated and swallowed. It should be mentioned here that about 98 per cent of the total body-calcium of the fish is concentrated in the bones. Metabolic studies with 'rice-fish' diets in which the bones were discarded showed that the subjects were in negative calcium balance (Basu *et al*, 1941, 1942).

The purpose of the present investigation was to determine whether the calcium and phosphorus of the bones of small fish, when incorporated in the diet, are nutritionally available. The problem is of considerable practical importance in view of the fact that the extreme cheapness together with the wide distribution and availability throughout the year of small fish bring these within the reach of the poor among whom the deficiency of calcium and the problem of removing it are more acute.

#### EXPERIMENTAL

Procedure and methods were the same as described in previous papers. Seventy grammes (moist basis) of small fish of different varieties, namely, chapla (*Clupea chapra*), mala (*Amblypharyngodon mola*), icha (*Palaemon carcinus*) and kakchi (*Conica sobona*), were used in place of milk (Basu *et al*, 1941) or betel-leaves (Basu *et al*, 1942) as a supplement to the typical calcium-deficient Indian diet consisting of rice, pulse, vegetables and small amounts of oil.

It must be mentioned here that *Palaemon carcinus* is not really a fish\* but it is considered as such by common people in many parts of India. The small varieties of *Palaemon carcinus* used in this investigation were taken *in toto*, i.e. with the outer shell and head.

#### RESULTS AND SUMMARY

Examination of the results presented in the Table shows that the calcium content of the basal diets was only about 200 mg. This was much below the requirement (Basu *et al*, 1941) and the subjects were in negative balance. The administration of about 70 g of fish (moist basis) augmented the dietary calcium by 577 mg on an average, of which about 70 per cent were retained.

The phosphorus content of the diets was about 1.010 g which satisfied the requirement (Basu *et al*, 1939, 1941). The addition of the said amount of fish increased the intake by about 637 mg of which about 50 per cent were retained.

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\* It is a small variety of prawn.

TABLE

*Effect of the addition of small fish to a poor rice diet on calcium and phosphorus metabolism*

Each period consisted of three days The subject took the basal diet for six days and the fish supplement was then given for another six days

Experimental subject	Diet	Supplement	Period	CALCIUM METABOLISM				
				Dietary Ca (mg) per day	Urinary Ca (mg) per day	Faecal Ca (mg) per day	Balance (mg)	Percentage of ingested Ca utilized
C C N	Rice diet	Nil 70 g Kakchi fish " "	P II	220	20	317	-117	
			P III	220+380=600	30	390	+180	
			P IV	600	30	440	+112	
			AVERAGE OF P III AND IV	600	35	420	+145	69
C C D	Rice diet	Nil 70 g Kakchi fish " "	P II	202	82	391	-271	
			P III	202+341=543	100	372	+71	
			P IV	543	60	331	+152	
			AVERAGE OF P III AND IV	543	80	351	+112	112

TABLE—*contd*

Experimental subject	Diet	Supplement	Period	CALCIUM METABOLISM					PHOSPHORUS METABOLISM					Dietary Ca/P
				Dietary Ca (mg) per day	Urinary Ca (mg) per day	Faecal Ca (mg) per day	Balance (mg)	Percentage of ingested Ca uti- lized	Dietary P (mg) per day	Urinary P (mg) per day	Faecal P (mg) per day	Balance (mg)	Percentage of ingested P utilized	
K R G	Rice diet	Nil 70 g Mala fish " "	P II	212	126	164	- 78		942	402	459	+ 81		1/4 4
			P III	212+640 =852	166	423	+ 203		942+732 =1,674	502	698	+474		
			P IV	852	123	330	+399		1,674	494	683	+497		
			AVERAGE OF P III AND IV	852	144	377	+331	64	1,674	498	690	+486	55	1/1 9
G. C N	Rice diet	Nil 70 g Mala fish " "	P II	184	79	149	- 44		1,003	548	402	+ 53		1/5 4
			P III	184+599 =783	112	346	+325		1,003+442 =1,445	612	574	+259		
			P IV	783	105	285	+393		1,445	602	517	+266		
			AVERAGE OF P III AND IV	783	109	316	+358	67	1,445	637	545	+263	48	1/1 8

G C N	Rice diet	N <sup>o</sup> l 70 g Chapla fish	P II	158	52	218	-112		854	342	408	+ 44		1/5 4
			P III	158+844 =1,002	60	593	+349		854+788 =1,042	390	851	+401		
			P IV	1,002	73	300	+539		1,042	470	780	+392		
			AVERAGE OF P III AND IV											
H P D	Rice diet	N <sup>o</sup> l 70 g Icha fish	P II	178	89	173	- 84		1,242	592	502	+148		1/6 9
			P III	178+658 =836	132	494	+210		1,242+588 =1,830	698	682	+450		
			P IV	836	101	520	+215		1,830	682	687	+401		
			AVERAGE OF P III AND IV											
				836	117	507	+212	45	1,830	690	685	+455	52	1/2 1



These results, therefore, prove that the calcium and phosphorus derived from bones of small fish, which are invariably taken when the latter are incorporated in diets, are nutritionally available

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# INVESTIGATIONS INTO THE BIOLOGICAL VALUE OF MILK PROTEINS

## Part I.

BY THE RAT-GROWTH METHOD\*

BY

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[Received for publication, March 31, 1942]

## INTRODUCTION

THOUGH studies of the biological value of proteins (including those from animal sources) of different nutrients have been carried out in India, no investigations have so far been made on milk proteins. Ranganathan (1940) estimated the supplementary values of cow milk, buffalo milk and goat milk on groups of growing rats maintained on a poor rice-eater's diet. The apparent reason why milk proteins have received scant attention from biochemists is that consumption of milk amongst the masses in this country is very small. However, it appears from a recent report on Agricultural Marketing in India (1941) that about 2,892 lakh maunds† of cow milk, 3,217 lakh maunds of buffalo milk and 180 lakh maunds of goat milk are annually produced in India. In the present study an attempt has been made to investigate the relative growth-promoting properties of the proteins present in the milks of the cow, goat and buffalo.

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\* Paper read before the Physiology Section of Indian Science Congress held at Baroda, January 1942

† 1 lakh maunds roughly equal 8,200,000 lb

## EXPERIMENTAL

The technique advocated by Osborne, Mendel and Ferry (1919) and Osborne and Mendel (1920) was followed with slight modifications. Young white rats bred in this Laboratory and weighing on the average about 50 g were employed in assessing the relative rates of growth. As facilities for drying milk on a large scale did not exist and since fresh boiled whole milk is usually consumed as such, fresh samples of milk were obtained every day and added to the artificial food mixture, the latter being more stable was prepared from time to time and preserved in a refrigerator. The protein content of the samples of milk was determined daily and the fat and ash content (to estimate calorie value) determined once every week. The result of analyses (based on average) is shown in Table I. After adding the requisite quantity of milk to the artificial food mixture consisting of carbohydrates and fats, the food for each animal was heated over a water-oven to the consistency of a paste and given in a feeding-cup. Every effort was made to serve the food to individual animals in such quantities that the amount left over daily in the cups was small. On successive days food was distributed over the residue in the feeding-cup and then heated as usual. The weekly residue was collected separately for each animal by carefully scraping out the feeding-cup. Aliquot portions were analysed for nitrogen and protein ( $N \times 6.25$ ). The average composition of the food given to each group of animals is shown in Table II. Owing to the high fat content of buffalo milk a part of the fat had to be skimmed off in the Laboratory for use in the experiment in which protein was supplied at a 15 per cent level. The average composition of the skimmed milk thus obtained is shown in Table I —

TABLE I

*Analyses of the different varieties of milk (grammes per cent)*

	Moisture	Protein	Ether extractives	Ash.	Carbo hydrates
Cow milk	87.93	3.30	3.80	0.71	4.26
Goat milk	85.20	3.71	5.60	0.80	4.69
Buffalo milk (pure)	83.20	3.57	6.24	0.72	6.27
Buffalo milk (skimmed)	85.48	3.62	2.91	0.72	7.27

TABLE II

*Average composition of food supplied to the animals in grammes (approximate caloric value 2,500)*

Food mixture	COW MILK		GOAT MILK.		BUFFALO MILK	
	15 per cent level	10 per cent level	15 per cent level.	10 per cent level.	15 per cent level.	10 per cent level
Fresh whole milk	2,770	1,848	2,480	1,651	2,527	1,708
Corn starch (nitrogen free)	107.3	182.4	50.0	148.0	113.0	146.0
Pure sugar cubes (Taiko)	54.0	54.0	26.5	54.0	54.0	54.0
Coco nut oil	Nil	32.2	Nil	28.0	Nil	Nil
Calcium carbonate	6.0	6.0	6.0	6.0	6.0	6.0
Salt mixture	24.0	24.0	24.0	24.0	24.0	24.0

In addition each animal was given daily 3 drops of cod-liver oil and 2 c.c. of a 1 per cent solution of Marmite to provide the different vitamins. The nitrogen present in the Marmite solution was estimated and added to the protein intake figures. The amount of protein obtained from Marmite was insignificant as compared to the total intake. The total intake of protein was calculated from the amount supplied in the food *minus* the amount left in the residue at the end of the week for each individual animal. The animals were weighed and the experiment was continued for 8 weeks. Each animal was kept in a separate metabolism cage and the meal served in an enamel feeding-cup and water supplied from inverted glass-bulbs for one week previous to the beginning of the experiment so as to accustom the animals to their surroundings and food. There were six animals in each of the experimental groups. The experiments were carried out with the level of protein intake at 10 per cent and 15 per cent, in the case of the three kinds of milk. In the case of cow milk a third experiment was tried in which milk was supplied at a 5 per cent level of protein intake, but all the animals failed to put on weight during three consecutive weeks and the experiment was abandoned. Terroine (1936) has compiled a table showing the minimum protein content of diet which permits of growth in the rat and

according to this table no growth is possible with milk below the level of 6 per cent intake of protein

### RESULT

The arithmetic mean of the intake of protein and the gain in weight in grammes have been calculated for each group. Tables III, IV and V show the results for each animal at the end of 4 and 8 weeks respectively. The amount of protein obtained by each animal from Marmite at the end of 4 and 8 weeks was found to be 0.22 g and 0.43 g respectively. Consequently, the amount of non-milk protein quota in the diet as compared with the intake of total protein was negligible and its effect on growth can be neglected.

TABLE III

*Protein (cow milk) ingested and gain in weight*

Serial number of rat	Initial weight, g	AFTER 4 WEEKS				AFTER 8 WEEKS			
		* Food intake, g	Protein intake, g	Gain in weight, g	Biological value	Food intake, g	Protein intake, g	Gain in weight, g	Biological value

*At 15 per cent level of protein intake*

C1	49	191.6	33.16	69.0	2.08	376.9	64.31	102.0	1.59
C2	47	189.3	31.83	67.0	2.10	368.7	61.91	90.0	1.45
C3	43	158.8	27.11	54.0	1.99	312.4	52.41	73.0	1.39
C4	42	180.5	30.25	76.0	2.51	398.0	66.56	111.0	1.67
C5	44	187.9	31.47	71.0	2.25	403.6	67.05	104.0	1.55
C6	54	190.1	32.58	63.0	1.93	410.4	70.36	116.0	1.65

*At 10 per cent level of protein intake*

C7	47	143.3	16.54	40.0	2.42	285.8	32.50	59.0	1.82
C8	49	191.3	22.02	61.0	2.77	369.5	42.51	86.0	2.02
C9	47	150.6	17.72	41.0	2.31	307.9	35.20	66.0	1.88
C10	49	161.3	19.05	46.0	2.41	314.9	36.01	58.0	1.61
C11	47	166.3	19.59	51.0	2.60	312.8	35.90	65.0	1.81
C12	53	184.3	22.06	48.0	2.18	358.1	40.84	63.0	1.54

\* Expressed as dry weight

TABLE IV

*Protein (goat milk) ingested and gain in weight*

Serial number of rat	Initial weight, g	AFTER 4 WEEKS				AFTER 8 WEEKS			
		Food intake, g	Protein intake, g	Gain in weight, g	Biological value	Food intake, g	Protein intake, g	Gain in weight, g	Biological value

*At 15 per cent level of protein intake.*

G1	50	216.8	47.04	58.0	1.23	485.1	108.78	75.0	0.69
G2	51	207.0	47.72	68.0	1.42	474.0	112.00	103.0	0.92
G3	54	230.7	51.70	67.0	1.30	514.8	119.35	97.0	0.81
G4	50	219.2	48.67	61.0	1.25	482.4	111.60	77.0	0.69
G5	49	245.1	54.28	68.0	1.25	513.7	117.99	94.0	0.80
G6	50	214.6	47.29	47.0	0.99	467.7	109.45	69.0	0.63

*At 10 per cent level of protein intake*

G7	48	150.2	20.93	24.0	1.15	364.7	50.82	45.0	0.89
G8	48	236.6	34.19	74.0	2.16	514.7	75.91	89.0	1.17
G9	46	185.1	27.51	47.0	1.71	421.9	63.05	65.0	1.03
G10	47	229.1	33.79	61.0	1.81	496.7	74.90	76.0	1.01
G11	44	228.1	34.31	67.0	1.95	506.1	76.02	91.0	1.20
G12	44	203.0	27.72	44.0	1.59	441.3	64.28	59.0	0.92

TABLE V

*Protein (buffalo milk) ingested and gain in weight*

Serial number of rat	Initial weight, g	AFTER 4 WEEKS				AFTER 8 WEEKS			
		Food intake, g	Protein intake, g	Gain in weight, g	Biological value	Food intake, g	Protein intake, g	Gain in weight, g	Biological value

*At 15 per cent level of protein intake*

B1	51	128.3	22.40	32.0	1.43	262.3	44.84	51.0	1.14
B2	45	112.9	19.48	37.0	1.90	247.3	41.83	60.0	1.42
B3	42	157.0	27.40	63.0	2.30	313.9	54.15	86.0	1.59
B4	53	162.2	28.39	53.0	1.87	347.9	59.86	99.0	1.66
B5	50	124.3	21.68	43.0	1.98	255.9	43.68	62.0	1.42
B6	41	144.5	25.03	49.0	1.96	289.7	49.52	68.0	1.37

*At 10 per cent level of protein intake.*

B7	47	125.1	17.77	42.0	2.36	279.5	39.45	79.0	2.00
B8	52	202.7	33.19	80.0	2.41	516.0	65.16	119.0	1.83
B9	49	116.7	16.47	36.0	2.19	260.2	36.62	68.0	1.86
B10	50	143.6	19.62	48.0	2.45	307.1	42.90	87.0	2.03
B11	49	146.5	20.24	48.0	2.37	315.2	44.13	97.0	2.19
B12	48	236.5	30.45	70.0	2.30	501.1	63.68	120.0	1.88

## DISCUSSION ON THE FINDINGS

In comparing the relative growth-promoting capacities of the proteins in different foods, the growth index or biological value of the proteins of each food is represented by an index which has been defined as the amount of growth per gramme of protein of each of the foods consumed at similar levels of concentration (Osborne *et al*, 1919, Osborne and Mendel, 1920) In the last columns of Tables III, IV and V the biological values of cow, goat and buffalo milks at 10 and 15 per cent levels of protein intake have been shown The arithmetic mean (average) for each of the group of six animals is given in Table VI The table shows that all three kinds of milk have given a higher biological value at a 10 per cent level of intake than when given at the 15 per cent level The differences have been tested statistically and have been found to be significant in the case of each of the three milks (cow, goat and buffalo) According to 'student's' test, 'P' was found to be beyond the 0.01 level in the case of goat and buffalo milks and between 0.02 and 0.05 in the case of cow milk Basu, Nath and Gham (1936) and Basu, Nath and Mukherji (1937) have found that a 15 per cent level of protein intake is superior to the 10 per cent level in the case of green gram, lentil and khesari, whereas in the case of soya bean and field-pea the reverse was the case

TABLE VI

*Average biological values*

Source of milk protein.	AVERAGE GROWTH (G) PER G OF PROTEIN INTAKE			
	AFTER 4 WEEKS		AFTER 8 WEEKS	
	10 per cent level.	15 per cent level	10 per cent level	15 per cent level
Cow	2.45	2.14	1.78	1.55
Goat	1.73	1.24	1.04	0.76
Buffalo	2.35	1.91	1.97	1.43

Comparison of the biological values of the three kinds of milk after 4 and 8 weeks indicates that goat milk has the lowest nutritive value at both 10 and 15 per cent levels of intake When cow and buffalo milks after the four-week period of growth are compared, the former appears to be superior to the latter at both levels of protein intake, but after the eight-week period the buffalo milk appears



to be superior to the cow milk at a 10 per cent level, whereas at a 15 per cent level of intake the cow milk gives a superior value. But this apparent anomaly completely disappears after further analysis of the figures. By the 't' test it is found that the differences between the mean biological values of cow and buffalo milk is not real, i.e. such differences could have arisen by chance alone.

TABLE VII

*Comparison of mean biological values after eight-week periods by the 't' test*

Milk proteins under comparison		Value of 't'	Level of significance 'p'	REMARKS*
10 per cent level	Cow and goat	8.324	Below 0.01	Significant
	Cow and buffalo	2.086	Between 0.1 and 0.05	Not significant
	Goat and buffalo	12.25	Below 0.01	Significant
15 per cent level	Cow and goat	12.600	Below 0.01	Significant
	Cow and buffalo	1.376	Between 0.2 and 0.1	Not significant
	Goat and buffalo	7.773	Below 0.01	Significant

\* n (degrees of freedom) = 10 in each case

### SUMMARY

The comparative nutritive values of the proteins of cow, goat and buffalo milks were assayed by the rat-growth method. With each of these milks a 10 per cent level of protein was found to be more favourable for growth than a 15 per cent level. The differences were tested statistically and found to be significant.

The growth-promoting capacity of goat milk per unit amount of protein present was found to be significantly inferior to that of cow or buffalo milk. No significant difference could be found between the proteins of cow or buffalo milk.

### ACKNOWLEDGMENTS

The authors are obliged to Mr S Chatterjee of Bacteriophage Laboratory for kindly looking after the animals and to Lieut-Colonel S L Mitra, I M S, and

Rai Bahadur Dr B P Mozoomdar, the successive Directors of Public Health, Bihar, for their interest in the work and encouragement

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## CASEIN AND LACTALBUMIN OF ASS MILK

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[Received for publication, March 31, 1942]

VALUABLE work has been done on cow-milk casein and lactalbumin, but comparatively little attention has been paid to the corresponding proteins of the milk of other mammals. In this connection the detailed investigations of human- and goat-milk caseins by Bosworth and van Slyke (1915) and Plimmer and Lowndes (1937) require mention. Although the general composition of the milk caseins have received attention, a systematic study of the casein from ass milk has not been made. The present study was undertaken to throw light on the nature of the casein in ass milk and to compare it with the casein of the milk of other species. In a preliminary paper (Anantakrishnan, 1941) the composition of ass milk has already been reported. In this investigation the preparation and analysis of the casein and lactalbumin of ass milk has been undertaken and an attempt has been made to study the enzymic degradation of ass-milk casein and to compare the results with those obtained with cow-milk casein.

### PREPARATION OF ASS-MILK CASEIN

The method adopted was that of van Slyke and Baker (1918). One litre of milk pooled from four asses was used and acid precipitation was carried out on the skimmed samples. The final pH was adjusted to 4.6 by means of a glass-electrode. The casein was powdered to pass through a sieve of 60 meshes per square inch and stored *in vacuo* over sulphuric acid. The yield of the casein on an average was 5.4 g per litre of the milk. Nitrogen was determined by the micro-kjeldahl procedure, sulphur by the method of Masters (1939) and

phosphorus by that of Fiske and Subbarow (1925) The analysis of five samples of casein is shown in Table I —

TABLE I  
*Proximate analysis of ass-milk casein*

Sample number	Ash, per cent	Nitrogen, per cent	Phosphorus, per cent	Sulphur, per cent
1	0 12	15 12	1 01	0 54
2		15 43	0 99	0 54
3		14 76	0 89	0 58
4	0 19	15 32	0 96	0 51
5		15 06	0 95	

The phenolphthalein equivalent, formal titrable nitrogen and specific gravity of the milk casein are presented in Table II —

TABLE II  
*Phenolphthalein equivalent, formal titrable nitrogen and specific gravity of ass-milk casein*

Sample number	Ml of 0 1N alkali required to neutralize 1 g of casein	Formal titrable nitrogen in ml of 0 1N alkali	Specific gravity at 22°C
1	9 23	2 10	1 32
2	9 10	2 15	
3	10 60	2 72	1 20

Number 3 was a sample from cow milk prepared by the van Slyke and Baker (*loc cit*) method, which was used wherever necessary in the present investigation Though it is known that the density of caseins varies with the method of preparation, it is interesting to note that ass-milk casein is a little denser than cow-milk casein, when prepared under identical conditions

#### THE NITROGEN DISTRIBUTION OF ASS-MILK CASEIN

The need for accurate information as to the quantities of the amino acids yielded on the acid hydrolysis of proteins, in general requires no emphasis in view

of the importance of amino acids in nutrition. In the present investigation the van Slyke method was used with the following modifications —

- (i) The amide nitrogen was determined by distillation in the Parnas Wagner apparatus for  $4\frac{1}{2}$  minutes (Damodaran, 1931)
- (ii) The dicarboxylic acid nitrogen was also determined according to the method of Damodaran (*loc cit*)
- (iii) The basic phosphotungstate was dissolved in sodium hydroxide solution and the phosphotungstic acid removed by means of barium chloride. This final solution of the bases was used for the determination of arginine by boiling with 40 per cent sodium hydroxide (van Slyke). Arginine was also determined independently on a separate aliquot of the hydrolysate of the protein by the Hunter and Dauphinee (1929-30) method after the removal of the amide nitrogen, and also by boiling with 40 per cent sodium hydroxide solution
- (iv) The histidine value was calculated in the usual way
- (v) Cystine could not be estimated in the basic fraction. It was determined on a separate hydrolysate by the methods of Folin and Lugg (1932) and Lugg and Sullivan (1933)
- (vi) Independent determinations of tyrosine and tryptophan were carried out by the colorimetric method of Folin and Marenzi (1929). Tables III and IV show the nitrogen distribution and the results of the independent determination of the amino acids —

TABLE III

*Nitrogen distribution of the various caseins expressed as percentage of total nitrogen*

Caseins	Cow	Human	Ass	
Amide N	11.70	11.82	10.87	
Humin N	0.68	0.79	0.93	
Dicarboxylic acid N			17.77	
Arginine N	7.77	7.11	8.52	9.40* 10.68†
Histidine N	2.61	1.96	5.38	
Leucine N	11.77	7.92	8.39	
Mono amino N	60.94	59.97	37.05	
Non amino N	6.41	9.24	9.72	

Values for human and cow milk caseins are taken from Plummer and Lowndes (*loc cit*)

\* Represents the arginine nitrogen on an independent hydrolysate by the Hunter and Dauphinee (*loc cit*) method

† Represents the arginine nitrogen on an independent hydrolysate of the protein by the van Slyke method

TABLE IV

*Tyrosine and tryptophan in ass-milk casein*

Sample number	Protein	Tyrosine, per cent	Tryptophan, per cent
i	Casein	4 00	0 392
ii	Casein	4 09	0 350
iii	Hammarsten's casein	5 05	1 290
iv	Casein (baryta hydrolysis)	4 12	0 430

Since the figures obtained for tryptophan of ass-milk casein are low when compared with other milk caseins, it was thought that the alkali may have destroyed the amino acid to a great extent. Baryta was therefore used as the hydrolytic agent. Table IV shows that the values given by the alkali and baryta hydrolysis agree closely, confirming the low tryptophan content.

From Table III it is clear that there is not much difference in the values for amide or humin nitrogen in the three caseins. The dicarboxylic acid nitrogen of the ass-milk casein is 17.77 per cent, whereas a value of 15.47 has been reported for cow-milk casein (Damodaran, *loc cit*). Arginine nitrogen is comparatively higher in ass-milk casein than the corresponding value for other milk caseins. The lysine nitrogen value of cow-milk casein is higher than that given by human- or ass-milk caseins. The non-amino nitrogen figures for the ass- and human-milk caseins closely agree, whereas in cow-milk casein the figure is very low.

Cystine nitrogen was not included in the scheme of nitrogen distribution as difficulty was experienced in the determination of cystine sulphur. A test for sulphur using nitro-prusside reagent was found to be negative. Cystine was therefore estimated on an independent hydrolysate of the casein by the method of Folin and Marenzi (*loc cit*) as modified by Folin and Lugg (*loc cit*) and also by the method of Lugg and Sullivan (*loc cit*). Decolorization was effected by Norit, a treatment which results in no loss of the total disulphide (Lee, 1933). The values for the cystine content are given below —

Cystine (Folin and Lugg)	0 107 per cent
„ (Lugg and Sullivan)	0 088 „

The value for cystine in ass-milk casein is lower than that obtained for cow- or human-milk casein.

The values obtained for histidine and lysine by the van Slyke method are generally higher than those obtained by the Vickery and Leavenworth (1928) method. The isolation method of Kossel and Kutscher (1900) as modified by Vickery and Leavenworth (*loc cit*) is most reliable for the determination of basic amino acids. This isolation method requires large amounts (50 g) of the test

material and laborious Block (1934) modified it so as to make it possible to use only small quantities of the proteins Tristram (1939) has made some changes in Block's method which was used in the present investigation for the determination of the basic amino acids of ass-milk casein The results are presented in Table V —

TABLE V

*The dibasic amino acids of ass-milk casein*

Sample number	Weight of arginine flavianate in g	Arginine N found (corrected for arginine silver + 1.1 mg N per 100 ml) expressed as percentage of total N	Percentage of amino acid in casein
			<i>Arginine</i>
1	0.3014	9.16	4.23
2	0.2886	9.10	4.18
3	0.2215	6.62	3.00
	<i>Histidine di-flavianate</i>	<i>Histidine N</i>	<i>Histidine</i>
1	0.2235	3.13	1.74
2	0.2201	3.18	1.77
3	0.2075	2.73	1.55
	<i>Lysine picrate</i>	<i>Lysine N</i>	<i>Lysine</i>
1	0.2651	5.17	4.07
2	0.2341	4.73	3.71
3	0.3321	6.08	4.92

Samples 1 and 2 are caseins from ass milk and sample 3 is Hammarsten's casein taken as a standard for analysis

The arginine value for the ass-milk casein obtained by the van Slyke and Tristram's method are 4.48 and 4.21 respectively For histidine the results obtained by Tristram's procedure are about one per cent lower (1.75) when compared to the values obtained by the van Slyke method (2.97) The lysine value (6.5 per cent) given by the van Slyke method is high in comparison with the value obtained by Tristram's method (3.89 per cent)

## ENZYMIC CLEAVAGE OF CASEIN FROM ASS AND COW MILK

A considerable amount of work on the enzymic digestion of protein has been carried out, more especially on casein, egg albumin and edestin Hunter and Dauphinee (*loc cit*) found that the arginine was rapidly liberated from casein on tryptic digestion, but with edestin the rate was much slower Gode and Sahasrabudhe (1929) subjected caseins from cow and buffalo milks to the action of pepsin and trypsin and found that the buffalo-milk casein was less readily digested Tyrosine, tryptophan and cystine have been found to be amongst the first to appear, and to be liberated completely in a relatively short time Ragins (1928)



found that after one hour's digestion with pancreatin, casein yielded 75 per cent of its tryptophan. It was therefore of interest to study the rate of liberation of some of the amino acids when mammalian caseins were subjected to enzymic hydrolysis in order to find out if any structural difference among the various caseins could be revealed.

*Hydrolysis of ass- and cow-milk caseins by trypsin* — A 2 per cent solution of the caseins was prepared in Sørensen's phosphate buffer of pH 7.7. The solutions were filtered and total nitrogen was determined on 5-ml aliquots of the solutions. One per cent Pfanstiehl's trypsin was also prepared in the same buffer. Thirty ml of casein solution were added to a series of six 50-ml standard flasks and maintained at 37°C. The trypsin solution was also maintained at 37°C and 3 ml of the enzyme solution were added to each flask. At definite intervals, a flask was taken and made to mark with 10 per cent trichloroacetic acid. After 10 minutes the contents were filtered. Total and amino nitrogen (formal titration on neutralized solutions) (Northrop, 1926) were determined in the filtrates. Table VI gives values for total as well as amino N in milligrams per 15 ml of trichloroacetic acid filtrates from cow- and ass-milk caseins —

TABLE VI  
*Tryptic digestion of cow- and ass-milk caseins*

Digestion time in minutes	COW MILK CASEIN				ASS MILK CASEIN			
	Total nitrogen		Amino N	Com- plexity	Total nitrogen		Amino N	Com- plexity
0	1.96	(7.1)	0.42	4.7	3.04	(14.2)	0.29	10.5
10	6.47	(23.3)	0.76	8.5	9.93	(35.0)	0.44	22.5
20	10.75	(38.7)	1.41	7.6	14.68	(51.7)	0.94	15.6
40	17.63	(63.4)	1.72	10.0	19.32	(68.0)	1.27	15.2
60	19.71	(70.9)	2.22	9.0	19.74	(69.5)	1.69	11.6
120	23.48	(84.4)	2.91	8.0	21.50	(75.7)	1.86	11.5
240	25.42	(91.4)	3.01	8.4	22.37	(78.8)	2.04	10.8
Total nitrogen in 15 ml of the reaction mix- ture	27.81				28.40			

Brackets refer to nitrogen expressed as percentage of total nitrogen in the reaction mixture.  
The term 'complexity' refers to the ratio of total nitrogen to amino nitrogen.

A study of Table VI reveals that during the course of tryptic digestion the casein from ass milk was brought into solution at a very much faster rate in the initial stages during the first 20 minutes. At the end of four hours the digestion figures shown in brackets in Table VI amounts to 79 per cent in the case of ass-milk

casein, while the corresponding figure for cow-milk casein is 91 per cent representing a higher digestibility for cow-milk casein. This is an unexpected result, which, at first sight, is not in harmony with the experimental fact (Bhagvat and Sreenivasaya, 1936) that ass milk is more easily digestible than cow milk. It has, however, been generally found by Bhagvat and her collaborator that the digestibility of the casein particle in its natural environment (milk) is different from the digestibility of the casein prepared from the milk. In the case of ass milk, probably this difference is very pronounced. The casein from ass milk has a remarkable tendency towards aggregation, the moment it is separated from its natural environment by acid precipitation.

Cow-milk casein, under the conditions of experiment, undergoes a higher degree of proteoclastic degradation and an average complexity of 9.0 for the hydrolytic products was attained in the course of the first two hours. Ass-milk casein, on the other hand, on digestion under similar conditions and during the same period, yields hydrolytic products whose average complexity amounts to 11.7.

The low digestibility (79 per cent in four hours) of the casein and the higher complexity (11.7) of the resulting products of hydrolysis, which characterizes the enzymic degradation of the ass-milk casein, as compared with the corresponding figures for cow-milk casein, points to a decisive difference in the nature of the two casein preparations.

*Tryptic liberation of tyrosine and tryptophan from ass-milk casein*—The colorimetric method of Rags (loc cit) was followed. Tryptophan was precipitated as the mercury salt and tyrosine was also determined on the filtrates at different intervals. Tryptophan was determined by the indirect vanillin-hydrochloric acid reaction. A blank containing no protein but trypsin was treated similarly. The results are presented in Table VII—

TABLE VII  
*Tyrosine and tryptophan in mg per 5 ml  
of the reaction mixture*

Time in hours	Tyrosine	Tryptophan
0.5	2.00	0.103
1.0	2.40	0.114
6.0	4.00	0.267
24.0	4.21	0.270
52.0	4.19	0.385
67.0	4.20	0.385
90.8		0.396
115.0		0.430
139.0		0.430

Table VII shows the rate of liberation of specific amino acids, such as tyrosine and tryptophan. The rate at which tryptophan gets split off from cow-milk casein

is higher than the rate at which tryptophan is liberated from ass-milk casein (Ragins, *loc cit*) After one hour about 25 per cent of the total tryptophan is liberated during the enzymic digestion of ass-milk casein During the same period, cow-milk casein, according to Ragins, yields 15 per cent of its tryptophan Further, equilibrium is reached in ass-milk casein at the end of 115 hours and the amount corresponds to 0.43 per cent of casein This is in agreement with the tryptophan content of ass-milk casein previously reported The low content of tryptophan is once again confirmed from these values It is to be observed that the tyrosine liberated reaches a maximum at the end of 24 hours in ass-milk casein

*Liberation of phosphorus from ass-milk casein by trypsin and pepsin*—During recent years, the problem of elucidating the position and mode of linkage of phosphorus in the casein molecule has received the attention of several investigators It is well established that the phosphoric acid group of the phosphoproteins, such as casein and isthulin, is not in the nature of an inorganic impurity but definitely constitutes an integral part of the casein molecule (Hammarsten, 1906) It was therefore of interest to determine the nitrogen-phosphorus ratio of the hydrolytic products of ass-milk casein when subjected to the action of trypsin and pepsin It was thought that the results might reveal differences in the composition and character of the phosphopeptides The method employed was similar to that of Stirling and Wishart (1932) except that Pfannstiel's pepsin and trypsin were used The results of the tryptic and peptic digestion of the ass-milk casein are presented in Tables VIII and IX —

TABLE VIII

*Liberation of phosphorus and nitrogen during the tryptic digestion of ass-milk casein*

EXPERIMENT I			EXPERIMENT II		
10 ML OF THE REACTION MIXTURE CONTAINS 7.18 MG N AND 0.445 MG OF P			10 ML OF THE REACTION MIXTURE CONTAINS 14.51 MG OF N AND 0.898 MG OF P		
Time in minutes	Soluble N	Soluble P	Time in minutes	Soluble N	Soluble P
10	3.18	0.290	10	1.84	0.230
20	4.40	0.412	20	2.83	0.330
30	5.82	0.410	30	4.22	0.430
40	5.90	0.410	40	5.02	0.590
50	6.06	0.430	60	6.96	0.710
60	6.59	0.432	75	7.10	0.780
70	6.81	0.432	90	8.51	0.870
80	6.81		105	9.77	0.870
			120	9.56	

TABLE IX

*Liberation of phosphorus and nitrogen during the peptic hydrolysis of ass-milk casein*

Time in minutes	EXPERIMENT I		EXPERIMENT II	
	10 ML OF REACTION MIXTURE CONTAINS 9.25 MG OF N AND 0.56 MG OF P		10 ML OF REACTION MIXTURE CONTAINS 15.65 MG OF N AND 1.080 MG OF P	
	Soluble N	Soluble P	Soluble N	Soluble P
10	4.55	0.139	5.57	0.097
20	5.16	0.160	6.20	0.154
30	5.72	0.159	6.94	0.208
40	6.13	0.162	7.20	0.220
50	6.10	0.170	7.45	0.226
60	6.39	0.170	7.50	0.236
70			7.99	0.241
80			7.72	0.262
90			8.31	0.270
100			8.60	0.270

TABLE X

*Nitrogen-phosphorus ratios of trichloroacetic acid precipitate of the ass-milk casein during tryptic and peptic hydrolysis*

TRYPSIN EXPERIMENT			PEPSIN EXPERIMENT		
Time in minutes	I	II	Time in minutes	I	II
10	25.7	18.7	10	11.2	10.3
20		20.7	20	9.5	10.2
30	39.0	22.0	30	8.6	10.0
40	36.6	27.3	40	7.8	9.8
60		40.0	50	8.0	9.6
75		67.3	60	6.5	9.6
90		24.0	70		9.1
			80		
			90		9.1
			100		8.7

There are certain points of resemblance between the values presented above and those reported by Stirling and Wishart (*loc cit*). These confirm

the findings that the rates of attack of trypsin and pepsin are fundamentally different. As in the case of ass-milk casein, the rate of liberation of phosphorus (soluble) in the ass-milk casein is greater than that of acid-soluble nitrogen. It is interesting to note that with a low concentration of casein as substrate, all the phosphorus is rendered soluble, so that the nitrogen-phosphorus ratio of the acid-insoluble material rises rapidly. The results also confirm the findings of Stirling and Wishart (*loc cit*), and Herd (1936, 1937) that with pepsin acid-soluble nitrogen is liberated in preference to acid-soluble phosphorus. Naturally, the trichloroacetic acid insoluble residue contains a higher proportion of phosphorus.

#### LACTALBUMIN FROM ASS MILK.

Lactalbumin was prepared by the salting-out process using ammonium sulphate (Sørensen and Sørensen, 1939). A litre of milk was used at a time. Casein was precipitated by dilute acid, separated and washed twice. To the combined washings and centrifugate an equal volume of saturated ammonium sulphate solution was added. After 24 hours standing, the solution was centrifuged and the centrifugate stored in an ice-chest for subsequent treatment. This experiment was carried out for a period of seven days, with fresh lots of milk every day. The clear combined casein-globulin free solution (20.2 litres) was saturated with ammonium sulphate, and allowed to stand overnight. The solution was then filtered by gravity and the precipitate dissolved in a known volume of water. Saturated ammonium sulphate solution was added to bring the solution to half saturation and left overnight. The solution was filtered and the process repeated six times. After the third fractionation no globulin precipitate was observed. The final albumin precipitate was dissolved in 200 ml of water and subjected to an exhaustive dialysis in a continuous collodion dialyser apparatus (Sørensen, 1915-17), at a diminished pressure of 28 cm to 34 cm of Hg, for 12 days against distilled water in the cold. The albumin from the dialysed solution was precipitated with ice-cold 95 per cent alcohol. After drying in vacuum, the material was a feathery white powder easily soluble in water.

The lactalbumin of ass milk prepared in the above way gave the following analyses:  $N = 14.11$ ,  $S = 1.29$  and  $P = 0.14$  per cent.

*Nitrogen distribution of the lactalbumin from ass milk*—The nitrogen partition of the protein was carried out by the procedure adopted by Plimmer and Rosedale (1925). Tyrosine, tryptophan and cystine were also determined. The results are presented in Table XI.

These albumins differ in their histidine and lysine contents. The albumin of the human milk is higher in its lysine content than the albumins from cow and ass milks. As the material available was limited Tristram's procedure for dibasic amino acids was not followed.

TABLE XI

*Nitrogen distribution of the various lactalbumins expressed as percentage of total nitrogen*

Lactalbumins	Cow	Human	Ass		
Amide N	8.73	9.04	7.34		
Humic N	0.81	1.69	1.68		
Arginine N	7.23	10.62	10.15	11.14*	12.27†
Histidine N	3.95	1.20	2.43		
Lysine N	10.91	13.05	10.76		
Mono amino N	66.03	66.50	63.19		
Non amino N	2.65	2.01	2.02		

\* Represents the arginine nitrogen on an independent hydrolysate by the Hunter and Dauphinee (*loc cit*) method

† Represents the arginine nitrogen on an independent hydrolysate of the protein by the van Slyke method

The tyrosine, tryptophan and cystine content of the lactalbumin of ass milk are 2.92, 0.97 and 2.60 per cent respectively

### SUMMARY

Casein from ass milk has been isolated in a fairly pure state. On analysis the preparation has been found to contain a higher amount of phosphorus than the milk casein of other mammalian species. Arginine nitrogen is present in greater quantity in ass-milk casein than in cow-milk casein.

2. The enzymic digestion of the ass-milk casein has been carried out and the rate of liberation of tyrosine and tryptophan has been observed.

3. Casein from ass milk has been subjected to the action of trypsin and pepsin. The nitrogen-phosphorus ratio of the acid-soluble fraction, at different intervals of time, has been determined. The results were similar to those obtained from cow-milk casein.

4. The lactalbumin from ass milk has been prepared and analysed. A lower content of tryptophan, tyrosine and cystine and a higher value for lysine as compared with other lactalbumins characterizes the preparation.

### ACKNOWLEDGMENTS

The authors wish to express thanks to late Dr W. L. Davies, Director of Dairy Research in India, for criticisms and advice throughout this

investigation, and to Messrs M Sreenivasaya and B N Banerjee for interest and support

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# DISTRIBUTION OF BLOOD GROUPS IN A MADRAS POPULATION

BY

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[Received for publication, May 14, 1942 ]

THE publication of the following blood group figures from a Madras population may be of interest. Such a large number (2,334) has not been recorded from Madras so far as we are aware. The collection includes a few Mohammedans, Anglo-Indians and Indian Christians besides Hindus.

Samples of blood received for Wassermann and Kahn tests were utilized for grouping. The cells were separated by centrifugalization and were then washed with normal saline solution. A two per cent suspension of the cells was used for determining the group and macroscopic slide agglutination was the method adopted. Standard group sera were used.

The results are given in the Table along with figures for some of the tribes and communities of India published by various workers for comparison —

TABLE

Community	Total blood samples grouped	BLOOD GROUP				SNYDER'S FORMULA			Figures published by
		O	A	B	AB	P	q	r	
Hindus—Madras Presidency	1,834	39.2	24.42	30.2	4.84	16.68	20.23	62.61	Seshadrinathan and Timothy (present series)
Hindus—Calcutta	1,302	36.02	21.8	34.6	7.5	16.0	23.8	60.0	Groval and Chandra (1940)



TABLE—concl'd

Community	Total blood samples grouped	BLOOD GROUP				SNYDER'S FORMULA			Figures published by
		O	A	B	AB	P	q	r	
Hindus—U P	2,357	30.2	24.5	37.2	8.1	17.9	26.1	54.9	Malone and Lahiri (1929)
Mohammedans— Madras Presi dency.	141	31.24	28.4	38.34	2.13	16.59	22.79	55.9	Seshadramathan and Timothy (present series)
Mohammedans— Calcutta	321	29.5	24.6	36.4	9.3	18.8	26.5	54.3	Greval and Chandra (1940)
Anglo Indians— Madras	47	55.32	19.5	25.32	0				Seshadramathan and Timothy (present series)
Anglo Indians— Calcutta	346	37.2	37.8	19.3	5.4	24.8	13.4	60.8	Greval and Chandra (1940)
Anglo Indians— I M D Students	67	40.29	41.79	14.92	2.98	25.7	9.4	63.2	<i>Ibid.</i>
<i>Dravidians</i>									
Todas	200	29.5	19.5	38.0	13.0	17.8	30.0	54.3	Pandit (1934)
Santals—Chota Nagpur	589	24.3	27.5	36.8	11.4	21.8	28.0	49.3	Malone and Lahiri (1929)
Indian Christians— Madras	95	41.05	23.15	31.5	4.21				Seshadramathan and Timothy (present series)

It would be seen that very little difference in group distribution is noticed between the Hindus of Madras and the Hindus of Calcutta. Both O and B groups are higher than group A. Group O is found to be less among the Hindus of the United Provinces as compared with those of Madras and Calcutta. Greval and Chandra (1940) conclude from their data that there is practically no difference in the percentage distribution of blood groups between the Hindus and the Mohammedans of Calcutta. Our figures for Madras similarly show very little

difference between the percentage distribution between Hindus and Moham-medans The Anglo-Indians of Madras (the number tested is too small to be of value) have a higher B figure than the Anglo-Indians of Calcutta The higher percentage distribution of O and B groups in Madras is probably due to Dravidian and Aryan admixture

## ACKNOWLEDGMENT

We are indebted to Major K P Menon Officiating Director, King Institute, Guindy, for permission to publish these findings

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## VISCO-ELASTIC PROPERTIES OF UNSTRIATED MUSCLE

BY

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[Received for publication, March 10, 1942]

WINTON (1930) explained the viscous elastic properties of unstriated muscle by means of a triple mechanical model similar to the one for striated muscle (Gasser and Hill, 1924, Levin and Wyman, 1927). Neglecting the undamped elastic phase, the extension curves of unstriated muscle, according to Winton, are compounded of an exponential curve and a linear curve. Though no mathematical expression is given, the equation for such a curve would be of the form—

$$At + B \left( 1 - e^{-At} \right) \quad (1)$$

Now, this is precisely the equation for the motion of a body against resistance under a constant propelling force. It has been shown (Singh, 1938) that the extension of unstriated muscle is governed not only by a viscous resistance but also by the resistance offered by tone of the muscle, any substance that increases the tone of the muscle also diminishes the rate of extension and the terminal velocity of extension. This diminution in the rate of extension is not due to a viscous resistance, as the rate of shortening is increased, it is due to the force of contraction.

In this paper a simple mathematical treatment of the subject is presented.

If unstriated muscle extends with uniform velocity, then the forces causing or resisting the extension must be constant. The force of extension is gravity, while that of resistance is the viscosity and tone of the muscle. We may therefore assume tone to be a constant force as a first approximation, as it may vary with the length of the muscle. This is sufficiently accurate for our purposes, as only partial extension of the muscle is being considered here.

The extension of the undamped elastic system in unstriated muscle is so rapid compared with that of the damped system, that it may be considered to be instantaneous, producing an impulse, which imparts an initial velocity  $= v^0$  to the damped elastic system.

Neglecting the pure viscous system of Winton (*loc cit*), the extension of unstriated muscle reduces to the consideration of the extension of a damped elastic system, similar to that considered by Gasser and Hill (*loc cit*) for striated muscle. If the differences between the properties of striated and unstriated muscles are taken into account, then the postulation of a pure viscous system in the latter is unnecessary, and the model of Levin and Wyman (*loc cit*) serves for both.

The fundamental equation of motion of such a system is—

$$m \frac{d^2x}{dt^2} + k \frac{dx}{dt} + px = mg \quad (2)$$

where  $x$  = extension,  $t$  = time,  $m$  = the extending weight,  $g$  = gravity,  $k$  = coefficient of viscous or internal resistance,  $p$  = restoring force which is proportional to the extension

For unstriated muscle the equation would be of the form—

$$m \frac{d^2x}{dt^2} + k \frac{dx}{dt} + px = mg - T \quad (3)$$

where  $T$  = tone of muscle

Compared to striated muscle, unstriated muscle is very extensible, so that for partial extension  $p$  may be taken equal to zero. The equation of motion for unstriated muscle is therefore—

$$m \frac{d^2x}{dt^2} + k \frac{dx}{dt} = mg - T \quad (4)$$

The solution of this equation is—

$$x = (mg - T) t/k + \left( \frac{v^0}{k} - \frac{mg - T}{k^2} \right) (1 - e^{-kt}) \quad (5)$$

since the initial velocity is greater than the limiting velocity  $= V = \frac{mg - T}{k}$ ,  $v^0$  may be considered equal to  $\alpha + \frac{mg - T}{k}$ . Substituting this value in (5) the equation becomes—

$$x = (mg - T) t/k + \frac{\alpha}{k} (1 - e^{-kt}) \quad (6)$$

where  $\alpha = V - \frac{mg - T}{k}$

This equation (6) is similar to (1). The terminal velocity  $V = \frac{mg - T}{k}$ ,  
 $k = \frac{mg - T}{V}$

The equation for the release curve is—

$$m \frac{d^2x}{dt^2} + k \frac{dx}{dt} = T \quad . \quad . \quad (7)$$

$$x = \frac{T}{k} t + \frac{\alpha^I}{k} (1 - e^{-kt}) \quad . \quad (8)$$

where  $\alpha^I = V^I - \frac{T}{k}$  . . .

$k$  and  $T$  can be found by examination of one release and one stretch curve by the equation—

$$k = \frac{mg}{V + V^I} \quad (9)$$

or by using more than one stretch curve by varying the value of  $m$  Tone is

found by the equation  $T = \frac{mg V^I}{V + V^I}$  (10)

Using two stretch curves  $k = \frac{(m_1 - m_2)}{V_1 - V_2} g$

Where  $m_1$  and  $m_2$  are masses, and  $V_1$  and  $V_2$  the corresponding velocities In the Table are given some figures for  $k$  and  $T$  of *Mytilus* muscle from my previous papers in arbitrary units In every case where contraction has occurred, the value of  $T$  has increased

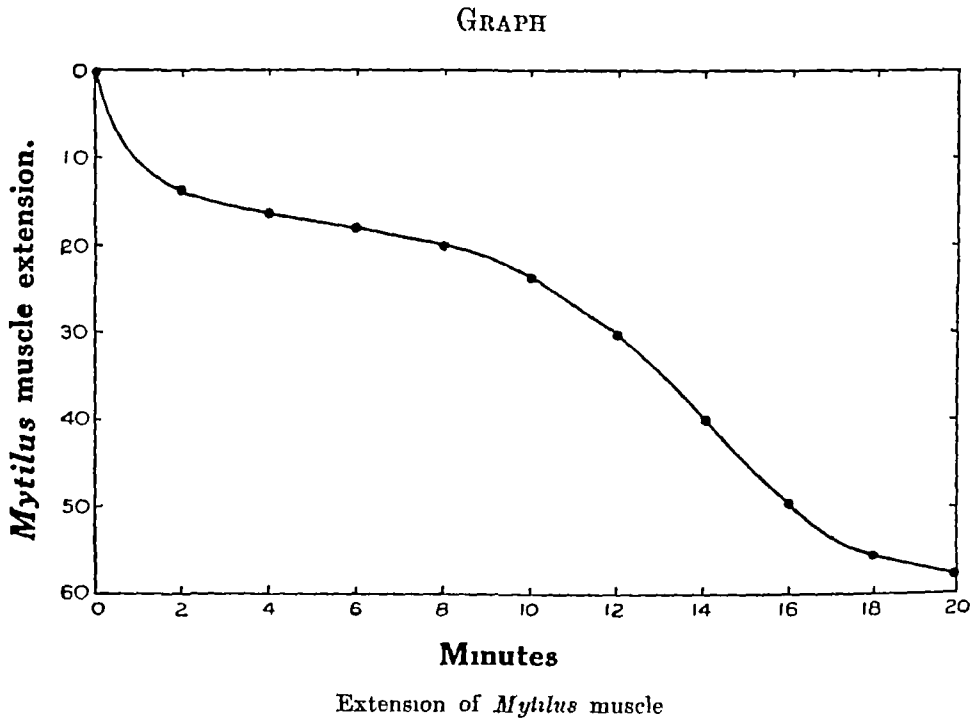
TABLE

*Effect of certain solutions on viscosity (k) and tone (T) of Mytilus muscle, units arbitrary*

Number of muscle	Control solution	$T$	$k$	Experimental solution	$T$	$k$
1	Sea water, pH 7.8	2.8	2	Sea water, pH 4.4	6.2	3.1
2	Sea water	7.04	1.1	Sea water with excess of magnesium	2.5	2.1
3	<i>Mytilus</i> saline	1.56	2.6	0.1 M KCL	3.6	5.04

The Levin-Wyman model is thus applicable to unstriated muscle, the damped spring is very weak. To introduce tone in the model, an electric current may be passed through the spring, which will make the spring contract. The attraction between the coils will vary inversely with the square of the distance. It is not

known what law governs the wrinkling of myosin molecules, but the behaviour of unstriated muscle is somewhat in accordance with a similar law. It will be seen from the Graph that the velocity of extension may increase prior to its final exponential diminution due to the boundary elastic system. A further examination of the subject will be presented in a later paper.



### SUMMARY

A mathematical equation for the extension of unstriated muscle is given, the Levin-Wyman model for striated muscle is also applicable to unstriated muscle, provided the differences between the properties of the two muscles are taken into account.

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THE EFFECT OF NEUROTOXIN, HÆMOLYSIN AND  
CHOLINE ESTERASE ISOLATED FROM COBRA  
VENOM ON HEART, BLOOD PRESSURE  
AND RESPIRATION

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[Received for publication, May 6, 1942]

VENOMS of snakes have been found to have different actions—neurotoxic, hæmolytic, etc —and poisons from different species have a preponderance of one or other of the principles. The various physiological effects of crude cobra venom have been studied by different workers. In order to ascertain which of the physiological effects is produced by which of the constituents of cobra venom, it is essential that the active principles of the venom should be isolated and their properties investigated separately. Ghosh, De and Choudhuri (1941) have succeeded in isolating from the crude cobra venom various enzymes together with neurotoxin and hæmolysin.

The following work was undertaken to determine the actions of some of the separated principles, especially neurotoxin, hæmolysin and choline esterase obtained from the venom of the Indian cobra (*Naja naja*) by Ghosh, De and Choudhuri (*loc cit*). The preparations were stored in desiccators kept inside a refrigerator and solutions were prepared just before use. It was found that the preparations lose their strength if kept dissolved even for 24 hours, which confirm the findings of Calmette and Massor (1914). The actions of the neurotoxin, the hæmolysin, the choline esterase and also of the crude cobra venom on the heart, the blood pressure



and the respiration were determined. The m.l.d. of the neurotoxin used was 0.021 mg. for the pigeon and that of the crude venom 0.1 mg. The sample of hæmolysin was weight for weight 11 times more effective than the crude venom.

THE ACTION OF THE NEUROTOXIN, THE HÆMOLYSIN, THE CHOLINE  
ESTERASE AND THE CRUDE COBRA VENOM

(1) *Effect on toad's heart* —The experiments were performed on about 50 toads, weighing between 70 g. and 100 g. The heart was exposed and a cannula carefully tied without damaging the sinus. Ringer-Locke solution, the pH of which was carefully adjusted to 7.6, was led into the heart, by a piece of rubber tubing from a Simm's cannula, the overflow tube of which was placed 4 inches above the heart. The perfusion pressure was thus kept constant at 4 inches of water throughout the

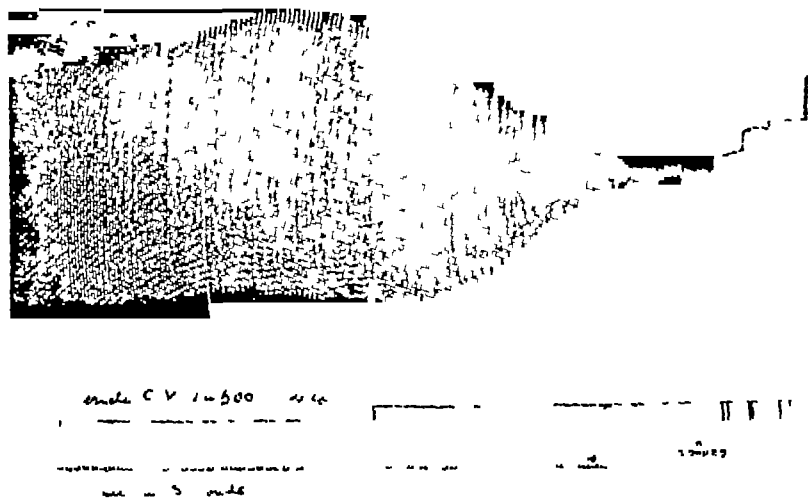


FIG. 1 —Effect of crude cobra venom (concentration 1/500)  
on toad's heart

whole series. A reservoir kept at a higher level supplied a steady stream of perfusion fluid into the Simm's cannula. Measured amounts of different strengths of the venom or of the separated principles dissolved in Ringer-Locke solution, were run into the Simm's cannula after simultaneously cutting off the supply of the normal perfusion fluid from the reservoir. When all the toxin solution had run in, this supply was replaced by Ringer-Locke solution for washing out the heart. In most cases the heart was perfused *in situ*, but in some of the experiments the heart isolated from the body was used. The results obtained in both the cases were similar.

Tables I to IV give the analysis of some of the typical results of perfusion with the different principles —

TABLE I

*Neurotoxin*

PERFUSED WITH RINGER LOCKE SOLUTION		PERFUSION WITH NEUROTOXIN						AFTER WASHING WITH RINGER LOCKE SOLUTION		
Height of contraction in inches	Heart beat per minute	Strength of dose	Quantity perfused in c.c.	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Heart beat per minute	Height of contraction in inches	Condition of heart
1.62	35	1 in 1,000,000	5	2.25	42	1.6	34	32	1.7	Regular
1.5	32	1 in 1,000,000	5	2.2	38	1.4	32	34	1.38	"
0.7	60	1 in 1,000,000	10	1.2	62	0.9	60	50	1.0	"
1.62	34	1 in 100,000	5	2.0	39	1.1	29	36	1.6	"
1.0	92	1 in 100,000	5	1.75	94	0.75	92	92	1.15	Slightly irregular
2.4	54	1 in 50,000	10	2.9	66	1.0	60	58	2.2	Regular
1.87	89	1 in 10,000	5	2.87	90	1.12 (Irregular)	88	88	1.7	"
1.4	78	1 in 1,000	4	2.5	94	0.8 (Irregular)	92	84	1.2	"

TABLE II

*Hæmolysin*

PERFUSED WITH RINGER LOOKE SOLUTION		PERFUSED WITH HÆMOLYSIN						AFTER WASHING WITH RINGER LOOKE SOLUTION		
Height of contraction in inches	Heart beat per minute	Strength of dose	Quantity perfused in c.c.	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Condition of heart
2.5	70	1 in 1,000,000	5	2.55	86	1.2	85	2.7	78	Irregular
0.8	48	1 in 1,000,000	5	0.9	46	0.6	60	0.8	56	Regular
1.0	70	1 in 1,000,000	5	1.0	70	0.9	70	1.2	68	Irregular
1.2	88	1 in 10,000	5	1.3	84	1.0	104	1.3	48	"
1.1	86	1 in 10,000	10	1.3 (Irregular)	78	0.5	92	1.0	74	"
1.9	~100	1 in 1,000	5	2.6 (Irregular)	100	1.5	106	1.8	80-42	"
1.5	100	1 in 500	2.5	2.6	84	1.0	120	2.3-1.7	96	"

TABLE III

*Crude cobra venom*

PERFUSED WITH RINGER LOCKE SOLUTION		PERFUSED WITH CRUDE COBRA VENOM						AFTER WASHING WITH RINGER LOCKE SOLUTION.		
Height of contraction in inches	Heart beat per minute	Strength of dose	Quantity perfused in c.c.	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Condition of heart
1.1	48	1 in 1,000,000	10	1.5	48	1.5	48	1.5	45	Regular
1.2	78	1 in 100,000	20	1.0	82	1.1	78	1.5	72	"
1.1	62	1 in 100,000	5	1.9	85	0.6	68	1.4	40	"
1.2	74	1 in 10,000	5	1.8	120	1.0	80	1.5	77	"
2.5	98	1 in 1,000	4	3.0	80	Irregular		2.0	80	Irregular
1.85	81	1 in 500	1	2.2	118	Systolic contracture				

TABLE IV  
*Choline esterase*

PERFUSED WITH RINGER LOCKE SOLUTION		PERFUSED WITH CHOLINE ESTERASE				AFTER WASHING WITH RINGER LOCKE SOLUTION		
Height of contraction in inches	Heart beat per minute	Strength of dose	Quantity perfused in c c	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Condition of heart
0.8	90	1 in 1,000	3.5	1.0	90	0.8	90	Regular
1.4	84	1 in 500	2.5	1.6	84	1.3	84	"
1.35	90	1 in 500	2.0	1.5	90	1.2	90	"

The augmentation of contraction of frog's heart with cobra venom was noted by Cushney and Yagi (1918), Basu (1927), Gautrelet, Halpern and Cortigiani (1934) and Gottdenker and Waschstein (1940). The subsequent enfeeblement of contraction and systolic contracture was also noted by Elliott (1904), Epstein (1930), Esawe (1933), Nakamura (1933, 1934), Kusnetow (1936), Gottdenker and Waschstein (*loc cit*). In our experiments with the crude cobra venom we obtained augmentation of contraction lasting for a short time, followed by a diminution in height of contraction to less than the normal in all concentrations (Table III). With higher concentration (1/10,000 or more) the heart became irregular with ventricular block, which disappeared on washing except with concentration above 1/1,000 when the block persisted. On perfusing the heart with venom concentration of about 1/500, after the initial stimulation,

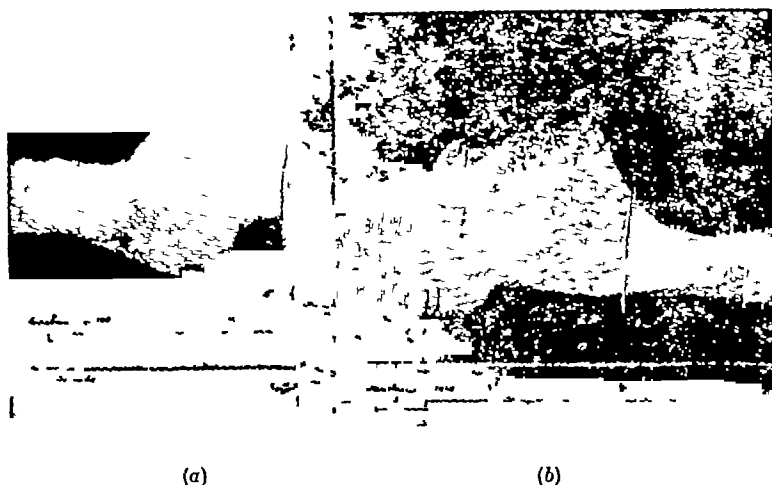


FIG. 2—(a) Effect of neurotoxin on toad's heart (1/100,000) (b) Effect of neurotoxin on toad's heart (1/1,000)  $\times$  Drum stopped for 1 minute

the heart stopped in systole which was not recovered from even after prolonged washing. The degree of reaction to each dose of crude cobra venom, of neurotoxin and of haemolysin differed in different toads, some being more sensitive or resistant than others.

Most of the effects of perfusion with neurotoxin are similar to that of the crude cobra venom (Table I). The main differences are that the auriculo-ventricular block is less evident and the systolic contracture of the heart is not obtained with neurotoxin. On perfusing with neurotoxin there is an immediate augmentation of both auricular and ventricular contractions, especially the latter. This is followed by diminution of contraction and in most cases the heart beating regularly. The heart beats irregularly if the concentration of the

neurotoxin is 1/10,000 or more, but on washing the beats become regular and approach the normal strength. No stoppage of heart with systolic contracture was obtained even with very concentrated solutions of neurotoxin. In some toads there was only a slight contracture for a few beats.

On perfusing with hæmolysin there is an augmentation of both auricular and ventricular contractions followed by marked diminution of contraction and irregular beating of the heart (Table II). This irregularity with auriculo-ventricular block was obtained in every case, even with a dilution of 1/1,000,000 and was not removed in most cases even after prolonged washing.



(a)



(b)

FIG. 3 —(a) Effect of hæmolysin on toad's heart (1/1,000,000) (b) Effect of hæmolysin on toad's heart (1/500) x Drum stopped

Perfused with strong solution of choline esterase (1/1,000 or more), the toad's heart showed slight augmentation of contraction (Table IV), which disappeared quickly after washing, the force and frequency of the heart remaining regular.

Gottdenker and Wachstein (*loc cit*) did not find any change in the frequency of the heart beat after perfusing with cobra venom. In our experiments with crude cobra venom we obtained increase in the heart rate during the augmentary period and with strong concentration the rate became irregular with group formations. On perfusing with neurotoxin no distinct change was observed in the frequency of the heart, there was only a slight quickening during the augmentary period. With hæmolysin, on the other hand, the heart rate increased with the diminution of the force of contraction and diminished again on washing. The heart also

became irregular with the ventricular block persisting for longer or shorter periods. With choline esterase there was no change in the frequency of the heart.

On repeatedly perfusing the heart with neurotoxin and hæmolysin, after washing with Ringer-Locke solution after each perfusion, it was found that the heart responded to each perfusion in the usual way, but the ventricular block persisted in the case of hæmolysin and appeared much later with neurotoxin. Table V shows the analysis of a typical result of repeated perfusions with neurotoxin.

(2) *Effect on mammalian heart*—The effects of the venoms on intact rabbits and guinea-pig's heart were also recorded, the animals being anaesthetized with urethane. After fixing the tracheal cannula the thorax was opened to expose the heart and the respiration was maintained by respiration pump. The movements of the heart were recorded graphically by attaching it to a lever. The different doses of the toxins were injected intravenously, dissolved in warm Ringer solution.

Gautrelet and Halpern (1934) obtained acceleration of the heart beat in dogs with cobra venom, while Feldburgh and Kellaway (1937*a*, 1938) found heart failure



FIG. 4—Effect of repeated dose of neurotoxin on toad's heart

with impaired conduction with doses of 0.2 mg to 2.0 mg per kilo. We did not find any effect on the heart with weak doses of neurotoxin (1.5 mg per kilo), but a pronounced and prolonged stimulation of contraction was obtained with bigger doses (6.0 mg per kilo). There was no change in the frequency and the beat was regular. A second effective dose produced further augmentation. Similar augmentary effects were obtained with hæmolysin and with strong dose (8 mg per kilo), the heart beat irregularly with periods of weaker or stronger contractions. The heart beat became progressively slower and the beats weaker and ultimately the heart stopped. The blood taken from the heart was found to have been partially hæmolysed. On injecting a small dose of crude cobra venom (0.5 mg per kilo) the heart stopped in a short time.

(3) *Effect on blood pressure and respiration*—Rabbits under urethane anaesthesia were used. The blood pressure was recorded by a mercurial manometer in the usual way and the respiration by Samson Wright's Respiration Recorder, which along with the rate also records the volume of air breathed. The preparations were introduced into the femoral or popliteal veins dissolved in 2 c.c. of warm Ringer solution.



TABLE V

Dose	Strength of dose	PERFUSED WITH RINGER LOCKE SOLUTION		PERFUSED WITH NEUROTOXIN		AFTER WASHING WITH RINGER-LOCKE SOLUTION		
		Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Height of contraction in inches	Heart beat per minute	Condition of heart
1st	1/100,000	1.5	34	2.2	42	1.5	34	Regular
2nd	1/1,000,000	1.5	34	2.0	42	1.5	31	"
3rd	1/1,000,000	1.5	31	2.1	37	1.5	31	"
4th	1/100,000	1.5	34	2.06 (Irregular)	39	1.05	28	"
5th	1/100,000	1.0	28	1.7 (Block)	35	1.0	28	Irregular
6th	1/4,000	1.0	28	1.9 (Irregular)	35	1.45	26	"

## RESULTS

The effect of cobra venom on circulation seems to depend on the dose. With small dose (2 mg in cats and rabbits) Epstein (*loc cit*) obtained a rise of blood pressure. The pressure effect with small doses was also obtained by Elliott (*loc cit*), Chopra and Iswariah (1931), Venkatachalam and Ratnagiriswaran (1934), Gottdenker and Wachstein (*loc cit*) using purified venom got lasting rise of blood pressure with doses of 6 micrograms to 9 micrograms per kilo. With doses of 0.25 mg to 0.5 mg per kilo in cats Chopra and Iswariah (*loc cit*) and Feldburgh and Kellaway (1937, 1937a, 1938) obtained a steep fall of blood pressure and death in a few minutes. Gautrelet, Halpern and Cortigiani (*loc cit*) also obtained fall of pressure in dogs with a dose of 0.1 mg. Amuchastagni (1939) obtained the same result in dogs with open thorax. In our experiments with crude cobra venom after a preliminary rise a sharp fall of blood pressure and stoppage of heart and respiration were obtained after injections of 2 mg of crude cobra venom per kilo.



FIG. 5—(a) Effect of neurotoxin on the blood pressure and respiration of rabbit. (b) Effect of a second dose of crude cobra venom on the blood pressure and respiration of the same animal after neurotoxin.

The effects of neurotoxin on the blood pressure of rabbits were found to be practically negligible, the dose ranging from 0.1 mg to 8.5 mg per kilo. There is usually a very slight gradual rise (2 mm to 5 mm of Hg). The most striking effect of neurotoxin is its action on respiration. On injection of 0.2 mg per kilo neurotoxin into a rabbit there is a slight rise of blood pressure and in 9 minutes the respiration gradually diminishes and stops altogether. When the respiration failed the blood pressure rose rapidly, due to asphyxia. On the administration of artificial respiration the blood pressure came back to the previous normal level or slightly lower and remained steady at this level so long as the artificial respiration was maintained. In some experiments the blood pressure was maintained thus for about 2 hours. With a dose of 1.25 mg neurotoxin per kilo the respiration stopped in 7 minutes, with 2.5 mg in  $4\frac{1}{2}$  minutes and with 8.3 mg in 2 minutes. A second dose of neurotoxin into a rabbit under artificial respiration, whose breathing had stopped as a result of previous dose of neurotoxin, showed the usual slight effect on blood pressure which remained steady. On the other hand, injection in the same way of crude cobra venom (2 mg per kilo) after neurotoxin into

rabbits under artificial respiration, caused a sharp rise of blood pressure followed by a rapid fall and stoppage of heart. Vago-sympathetic effects on the heart and the blood pressure were found to be negative after neurotoxin.

Injection of hæmolysin from 0.2 mg to 6 mg per kilo into rabbits produced, as obtained with neurotoxin, a slight gradual rise in blood pressure, but had no effect on respiration. Stimulation of vagus produced their usual effects on blood pressure, heart and respiration even after more than an hour. Blood was found to have been slightly hæmolysed (6 mg). With heavier dose about 10 mg per kilo both the circulation and the respiration failed in about a minute. In strong contrast to the action of neurotoxin, the administration of artificial respiration failed to maintain the heart and the blood pressure. On examination the blood was found to have been more than 50 per cent hæmolysed. After a dose of hæmolysin (4 mg)

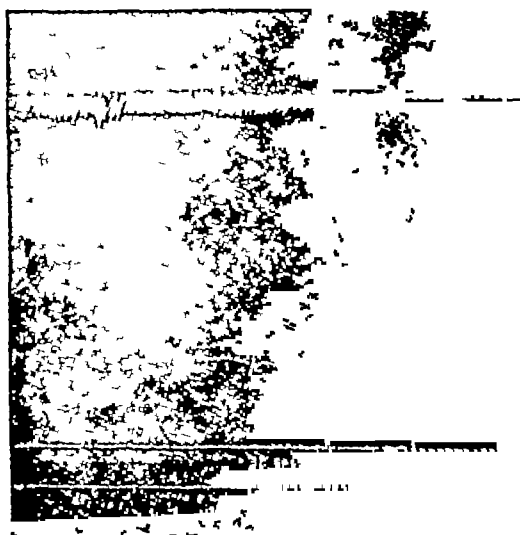


FIG. 6.—Effect of 10.6 mg of hæmolysin on a rabbit weighing 1.5 kg.

which was insufficient to cause the failure of circulation and respiration, injection of neurotoxin produced the usual effects and respiration stopped. On administration of artificial respiration the circulation remained unaffected, as was found with neurotoxin alone.

Administration of choline esterase up to 13 mg per kilo was found to have no action on respiration and caused only a slight rise in blood pressure.

From these experiments it is evident that with weak concentration of crude cobra venom the heart is stimulated, the force of contraction being especially affected. With higher concentration the stimulation is followed by depression with irregularity of the heart's action, and with high concentration of cobra venom the heart stops altogether in systolic contracture. These results confirm the work of other

authors already cited With small doses of crude cobra venom there is a rise of blood pressure, but with higher doses the circulation fails The crude cobra venom profoundly affects the respiratory movements, causing paralysis of respiration This has already been noted by Rogers (1903), Elliott (*loc cit*), Calmette and Massor (*loc cit*), Cushney and Yagi (*loc cit*), Kellaway (1933), Chopra and Iswariah (*loc cit*) and Venkatachalam and Ratnagiriswaran (*loc cit*)

Of the separated principles obtained from crude cobra venom, the neurotoxin has much the same effect on the heart as the crude cobra venom, but differs markedly in one respect The heart does not stop beating even with a high concentration of neurotoxin as it does with crude cobra venom The failure of neurotoxin to stop a beating heart is also seen in the rabbit and in the guinea-pig with intact circulation, when in response to injections of neurotoxin the heart goes on beating strongly No distinct effect was found on the blood pressure of rabbits after injection of neurotoxin, pressure remaining steady at about the normal level provided the respiration which had failed was maintained artificially The factor present in crude cobra venom which is responsible for heart failure is absent in the purified neurotoxin

The effects of administration of hæmolysin are complicated by the fact that it also causes hæmolysis of blood In the amphibian heart along with the stimulating and inhibitory effects there develops a persistent ventricular block This is well seen in hearts damaged by repeated injections of small doses of hæmolysin This was also seen in the rabbit and the guinea-pig's heart with intact circulation Small doses of hæmolysin have practically no effect on circulation and do not cause any change in the respiratory movements, but with high concentration there is failure of both

Thus, there is failure of circulation as the result of administration of crude cobra venom and of high doses of hæmolysin The circulatory failure after hæmolysis is probably due to its hæmolytic activity, as it is only evident with high doses when there is considerable hæmolysis There is also no stoppage of the perfused toad's heart even with strong solution of hæmolysin The factor responsible for the stoppage of heart and circulatory failure with crude cobra venom may be present in fractions not removed as neurotoxin or as hæmolysin or as choline esterase Other factors, such as liberation of histamine (Feldburgh and Kellaway, 1937b, 1938), action of lysolecithin (Feldburgh and Kellaway, 1938, Feldburgh, Holden and Kellaway, 1938), may be also contributory No attempt was made to clear up these problems, which will be dealt with in a subsequent communication

#### SUMMARY

1 Purified neurotoxin separated from crude cobra venom causes in the perfused toad's heart principally augmentation of the force of the heart beat With a concentrated dose or with repeated small doses there is irregularity with ventricular block which disappears with the washing of the heart Neurotoxin also stimulates the rabbit's or the guinea-pig's heart with intact circulation Unlike

crude cobra venom it does not cause stoppage of the heart, even in high doses in the toad, the rabbit or the guinea-pig

2 Neurotoxin has no definite action on the blood pressure of rabbits. The principle present in crude cobra venom which affects the blood pressure and causes cardiac failure is absent in neurotoxin.

3 Neurotoxin paralyses the respiratory movements and is the factor responsible for this effect of crude cobra venom.

4 In the perfused toad's heart hæmolysin produces augmentation and depression and irremovable irregularity and ventricular block without causing cardiac failure.

5 Hæmolysin in weak doses does not affect the circulation or the respiration but in heavy doses causes failure of both.

6 Choline esterase in high concentration slightly stimulates the perfused toad's heart but has practically no action on the blood pressure or, on respiration of rabbits.

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## STUDIES ON TRYPSIN-KINASE FROM BLOOD AND ON ENTERO-KINASE

BY

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[Received for publication, June 25, 1942]

THE AUTHOR (Iyengar, 1942a) has presented evidence for the presence of a trypsin-kinase in platelets capable of liberating trypsin from the trypsin-inhibitor compound existing in blood plasma. It is well known that platelets contain thrombo-kinase which catalyses the formation of thrombin from prothrombin. Ferguson (1939) has demonstrated similarity between thrombo-kinase and trypsin in so far as the latter reacts with prothrombin to produce thrombin. This similarity of thrombo-kinase and trypsin is limited only to their catalysing effect on blood coagulation. Iyengar (1942b) has extended this idea and adduced experimental evidence to the hypothesis that plasma trypsin possibly performs the function of the physiological thrombo-kinase.

The concomitant presence of trypsin-kinase and thrombo-kinase in platelets and the similarity of trypsin and the thrombo-kinase in certain respects led the author to investigate the possibility of thrombo-kinase acting also as trypsin-kinase on plasma proteins precipitated by acetone. In order to study this, it is necessary to obtain another source which contains thrombo-kinase. Russell's viper venom, on account of its powerful thrombo-plastic activity, is regarded as a good source of thrombo-kinase. Although this venom contains trypsin also in small quantities, its tryptic activity is practically negligible in high dilution (1 in 10,000 or 1 in 20,000) in which it is used for demonstrating its thrombo-plastic property. If the thrombo-kinase in Russell's viper venom can be shown to have the property of liberating active trypsin from the trypsin-inhibitor compound present in plasma this can be considered as evidence for the probable similarity between thrombo-kinase and trypsin-kinase. This trypsin-kinase should, however, not be confused with the entero-kinase which activates inactive trypsinogen of the pancreas.

The trypsin-inhibitor compound is obtained as usual by precipitating plasma with 4 volumes of acetone. By this process the whole of the plasma proteins are precipitated and along with this, the trypsin-inhibitor compound which is also a protein comes down. It is thus seen that this preparation contains a large quantity of protein, a small portion of which is the trypsin-inhibitor compound. The tryptic activity of this preparation is first determined in the usual manner. To a similar quantity of this preparation is added a solution of Russell's viper venom (1 in 10,000) the whole being suspended in a phosphate buffer of pH 8.4 and incubated for a period of 18 hours. The tryptic activity of this mixture is also estimated (Table I) —

TABLE I

Tryptic activity of	Increase in N P N , mg
1 g of the above preparation suspended in 20 c c of $\frac{M}{15}$ phosphate buffer of pH 8.4	1.61
1 g of the above preparation suspended in 20 c c of $\frac{M}{15}$ phosphate buffer of pH 8.4 to which is added 2 mg of Russell's viper venom (This amount brings the concentration of the venom in the digest to 1 in 10,000)	1.65

By incubating the Russell's viper venom with the plasma trypsin-inhibitor compound preparation, no significant increase in the release of N P N is produced. This result clearly demonstrates that the thrombo-kinase and the trypsin-kinase referred to by Schmitz and found by Iyengar to be present in platelets, are two independent entities.

The next question that arises is whether the trypsin-kinase found in the platelets and in fibrin is capable of activating trypsinogen from the pancreas and also whether the entero-kinase extracted from the intestinal mucosa can liberate trypsin from the trypsin-inhibitor compound present in plasma. It has been shown by Kunitz and Northrop (1935-36) that trypsinogen undergoes autocatalytic activation and this activation is further accelerated by the addition of trypsin. It is, therefore, necessary to obtain the kinase free from trypsin in order to study the activating effect on trypsinogen.

*Preparation of kinase from blood*—According to Schmitz (1936) fibrin clot contains both trypsin and the kinase. The trypsin can first be eluted by extraction with acetic acid and the kinase can then be eluted from the fibrin by extraction with alkali. By following this procedure the kinase extract, free from trypsin as far as possible, was prepared.

*Preparation of kinase-free trypsin*—Hog pancreas, immediately after killing the animal, is ground in a meat-chopper and dried with acetone and ether. It is

finally dried in air and powdered. A glycerol extract of this powder is used for the experiments.

*Preparation of entero-kinase*—The mucous membrane is scraped off, from the upper portion of the hog's intestine. It is shaken with 3 volumes of acetone and allowed to stand for 2 minutes. It is filtered and washed with acetone, then with a mixture of acetone and ether and finally with ether and dried in air and powdered. An aqueous extract of this powder was used for the experiments.

The trypsin-inhibitor compound from plasma was obtained by precipitation with acetone as reported in my previous communications on the subject.

*Action of kinase from blood on trypsinogen from pancreas*—A 1 per cent casein solution is prepared in  $\frac{M}{16}$  phosphate buffer of pH 8.4. Twenty c.c. of this solution is incubated with 5 c.c. of (1 in 10) glycerol extract of the kinase-free trypsin powder. The volume is then made up to 35 c.c. by the addition of water (mixture 1). To another 20 c.c. of the casein solution are added 5 c.c. of glycerol extract and 10 c.c. of the kinase solution obtained from blood fibrin. In this case the volume is already 35 c.c. (mixture 2). Twenty c.c. of the casein solution is taken in a third flask and mixed with 5 c.c. of glycerol extract and 10 c.c. of an aqueous extract of entero-kinase (mixture 3). All the three solutions are incubated for a period of 3 hours and the increase in non-protein nitrogen determined in each in the usual manner (Table II).—

TABLE II

Mixture	Increase in N.P.N., mg
(1) Casein + kinase free trypsin	2.5
(2) Casein + kinase free trypsin + kinase from fibrin	2.9
(3) Casein + kinase free trypsin + entero kinase	7.8

The kinase prepared from fibrin does not appear to activate kinase-free trypsin prepared from the pancreas.

Whether entero-kinase can liberate trypsin from the trypsin-inhibitor compound present in plasma, was next investigated. In this case, there was no necessity to supply any substrate since the trypsin-inhibitor compound prepared from plasma contains all the plasma proteins the autodigestion of which can be



The trypsin-inhibitor compound is obtained as usual by precipitating plasma with 4 volumes of acetone. By this process the whole of the plasma proteins are precipitated and along with this, the trypsin-inhibitor compound which is also a protein comes down. It is thus seen that this preparation contains a large quantity of protein, a small portion of which is the trypsin-inhibitor compound. The tryptic activity of this preparation is first determined in the usual manner. To a similar quantity of this preparation is added a solution of Russell's viper venom (1 in 10,000) the whole being suspended in a phosphate buffer of pH 8.4 and incubated for a period of 18 hours. The tryptic activity of this mixture is also estimated (Table I) —

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*Preparation of kinase-free trypsin* —Hog pancreas, immediately after killing the animal, is ground in a meat-chopper and dried with acetone and ether. It is

reported in this paper lend further experimental evidence in favour of the view put forward by Kunitz and Northrop. If the kinase prepared from blood fibrin can activate trypsinogen then the latter can be considered to be similar to the trypsin-inhibitor compound. From the results in Table II, it is apparent that the trypsinogen is not activated by the kinase from blood. It is therefore established that trypsinogen and trypsin-inhibitor compound are not identical. Conversely, it may also be concluded from the results obtained that kinase present in blood is different from the entero-kinase present in the duodenal mucosa.

It has been reported by Kunitz and Northrop that the addition of inhibitor to trypsinogen retards the activation of the latter by entero-kinase. A large quantity of the inhibitor will completely prevent activation. It is therefore clear that the inhibitor combines with trypsinogen and the resulting combination does not lend itself to be acted upon by entero-kinase. From these observations it can be inferred that the trypsinogen-inhibitor combination if complete, cannot be acted upon by entero-kinase. Whether the trypsin-inhibitor compound is acted upon by entero-kinase does not appear to have been previously studied. Such a study has been made in this paper and the results are given in Table III. It can be seen that trypsin has been freed from the compound by entero-kinase. While entero-kinase can thus split the trypsin-inhibitor compound, it appears that it is not capable of liberating trypsin from trypsinogen-inhibitor combination. It is interesting to note that the kinase from blood cannot perform the function of entero-kinase while entero-kinase has the property of splitting the trypsin-inhibitor compound, exhibiting a behaviour in this particular respect similar to that of the action of kinase from blood. It is likely that entero-kinase is a mixture of kinase (from blood) and some other factor which is responsible for the activation of trypsinogen.

#### SUMMARY

1 The relationship between thrombo-kinase and trypsin-kinase present in blood has been investigated. It has been found that thrombo-kinase and trypsin-kinase are two independent entities.

2 Blood-kinase does not activate trypsinogen from the pancreas and differs in this respect from entero-kinase.

3 Entero-kinase has been found to liberate active trypsin from the trypsin-inhibitor compound. It is therefore suggested that entero-kinase may be a mixture of a kinase which is similar to the blood kinase and another factor which can activate trypsinogen.

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TRANSMISSION OF INDIAN KALA-AZAR TO MAN BY  
THE BITES OF *PHLEBOTOMUS ARGENTIPES*,  
ANN AND BRUN.

BY

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[Received for publication, June 24, 1942]

PREVIOUS attempts to transmit Indian kala-azar to man have given uniformly negative results (Shortt, Craighead, Smith and Swaminath, 1928, Shortt, Craighead, Smith and Swaminath, 1929) and this failure left unforged a vital link in the chain of evidence incriminating *Phlebotomus argentipes* as the natural vector of the disease

It was felt by us that another attempt should be made to obtain this crucial evidence which would furnish the final scientific proof that *Phlebotomus argentipes* was the vector. With this object in view it was decided to initiate a new series of experiments to infect human volunteers with kala-azar through the bites of this insect

The general technique followed was that adopted by the Kala-azar Commission in previous attempts at human transmission but with one important change. This change lay in the technique of keeping the flies alive after the initial infecting feed on a kala-azar case. In previous experiments such flies were given feeds of blood on man or experimental animals to keep them alive until they were considered ready to feed on the human volunteer it was decided to infect. In the present series of experiments the flies, after the initial infecting feed on a kala-azar case, were kept alive on fruit juice until ready to feed on the human volunteer. The technique followed was that described by Smith, Halder and Ahmed (1940), who described successful transmission experiments with animals fed on by infected sandflies kept alive by this method until ready to infect (Smith, Halder and

Ahmed, 1941) The course of events in the case of each batch of laboratory-bred sandflies in the present investigation was as follows —

The flies were given an infecting feed on a case of kala-azar. They were then kept in tubes in an incubator at 28°C and, on the third or fourth day, transferred to lamp-globes where they were given a supply of raisins on which to feed. Here they were kept at the same temperature until the lapse of a minimum of eight days from the time of the initial blood meal. The mortality among the flies is high so that by the eighth day only 25 to 30 per cent may be available for feeding on the human volunteer to be infected. The most favourable survival rate is seldom over 50 per cent. The surviving flies of the batch were fed in a muslin cage on the volunteer, who was placed inside a net in an insect-proof room. The volunteer spent only one night in the laboratory and in the morning was sent back to a non-endemic area. The volunteers used were healthy Khasi males who had undergone a complete clinical medical examination, including X-ray examination of the lungs and examination of the urine, stools and blood. The Wassermann test and aldehyde test were negative. Syphilis, any lung lesion, albuminuria or general poor physique were considered sufficient reason for rejection. In addition, none of them had ever left the non-endemic area of the Khasi hills. The actual feeding was carried out at Gauhati, at the foot of the hills, where our laboratory was situated.

Five volunteers were utilized and, at the time of writing, three have become infected, the remaining two having not yet been examined\*. The details of the experiments are given in the Table.

The results shown in the Table leave no doubt as to the efficiency of *Phlebotomus argentipes* as a vector of the human disease in Assam but the question arises as to why transmission, so easily achieved in the present series of experiments, was not achieved in previous attempts. We do not propose now to discuss this point in detail but merely make two suggestions which will afford material for further study. The first of these suggestions is that in the present investigation there was the obvious difference in the method of keeping alive the flies after the infecting feed. Whether feeding them on fruit juice acts by increasing the virulence of the parasites or increases the heaviness of infections has yet to be investigated.

The second suggestion is one which has already been hinted at by Shortt, Smith, Swaminath and Krishnan (1931) who said, 'It is possible also, that the more rapid succession of passages of the parasites from man to fly and vice versa during an epidemic would enhance its virulence to a degree quite out of proportion to that possessed by it during non-epidemic periods. For this reason we believe that part of our previous lack of success in obtaining transmission was due to the fact that transmission experiments on a large scale with *Phlebotomus argentipes* and a really susceptible animal, such as the Chinese hamster or man, were only undertaken when the recent kala-azar epidemic was already on the wane and the virulence of *L. donovani* was already lessening.'

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\* See 'Note' at end of paper —Ed

TABLE  
Showing results of kala-azar transmission experiments with man

Name of the volunteer	Dates of feeds by infected flies	Total number of flies fed	Total number of flies found positive by dissection	Intensity of infection in positive flies	Interval between first feed on case and infective feed, days	REMARKS*
1	2	3	4	5	6	7
U Wollington Mason	20-12-41	0	3	2+++	0	First reported ill on 3-5-42 Sternal puncture on 28-6-42 ++ L D
	1-1-42	43	2	1+++	10-11	
	20-1-42	37	6	2+++	11-12	
	4-2-42	110	8	4+++	10-11	
				3+++		
				4+++		
				1+		
	20-2-42	16			10-12	
	17, 18-3-42	30	1	+++	9-12	
	30, 31-3-42	8			9-13	
	12, 13-4-42	28	1	+++	10-13	
	20, 21-4-42	47	7	1+++	9-12	
				3++		
	2, 3-5-42	53	10	3+	8-12	
				1+++		
				7+++		
				8++		

Explanation of markings in column 5 —

++++ = Extremely heavy infection of whole midgut and pharynx with flagellates so closely packed as to be immobile

+++ = Very heavy infection of midgut and pharynx

++ = Heavy infection of midgut and infection of oesophagus

+ = Infection of midgut

\* These results have been confirmed by cultures

TABLE—concl'd.

Name of the volunteer	Dates of feeds by infected flies	Total number of flies fed	Total number of flies found positive by dissection.	Intensity of infection in positive flies	Interval between first feed on case and infective feed, days	REMARKS*
1   U Saron Laloo	2	3	4	5	6	7
	27-12-41	48	8	6+++	9-10	First reported ill on 17-5-42
	9-1-42	55	1	2++	10	Spleen puncture on 31-5-42
	29-1-42	100	16	+ + + + +	10-11	+ L D
	9-2-42	126	7	14+++	9-11	
U Hth Challam	6-3-42	55	10	2+++	9-12	
				7+++		
				7+++		
				2++		
				1+		
	24-12-41	21	4	2+++	8-10	Did not report illness of any kind,
	6-1-41	28		1++	9-11	but when examined spleen noticed
	27-1-42	82	17	1+	10	to be 4 fingers on 8-6-42
	7-2-42	110	3	12+++		Spleen puncture on 12-6-42
	2, 3-3-42	32	6	3+++	10	L D
	25, 28-3-42	42		2++	9-10	
				1++	9-12	
				6++		

Explanation of markings in column 5 —  
++++ = Extremely heavy infection of whole midgut and pharynx with flagellates so closely packed as to be immobile  
+++ = Very heavy infection of midgut and pharynx  
++ = Heavy infection of midgut and infection of oesophagus  
+ = Infection of midgut  
\* These results have been confirmed by cultures

It is a fact that the previous series of experiments with man were commenced and carried on when the kala-azar epidemic had passed its peak and was on the down grade. If past history of the periodicity of kala-azar in Assam is to repeat itself this is the time when a new epidemic should be starting and it is possible that one factor at least in the genesis of epidemics may be increased virulence in the parasite. We do not wish to stress either of these points but put them forward for consideration and study.

### SUMMARY

Successful transmission of Indian kala-azar to man by the bites of *Phlebotomus argentipes* has been achieved in three out of five attempts.

### ACKNOWLEDGMENTS

In carrying out this investigation we have to acknowledge the great assistance received from many collaborators. Dr K C Halder, who was responsible for the diagnosis of cases and whose previous experience of the feeding technique was most useful, Dr S R Pandit, Director, Pasteur Institute and Medical Research Institute, Shillong, who performed the examinations on the infected volunteers, and Mr James John who was indefatigable in survey work and the collection of cases suitable for feeding of flies. To Dr Gordon Roberts of the Welsh Mission Hospital we are greatly indebted for his invaluable help in obtaining suitable volunteers. Lastly, we must acknowledge the self-sacrificing spirit of the human volunteers who submitted themselves for experimentation and helped in the final solution of a problem in tropical medicine of many years' standing.

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### Note

Since the above paper was submitted for publication the two remaining volunteers have developed kala-azar. Transmission by the bite of infected sandflies has thus been accomplished successfully in 100 per cent of cases (11th August, 1942).





## EDITORIAL NOTE

### THE TRANSMISSION OF KALA-AZAR

THE recrudescence of kala-azar in severe epidemic form in Assam and certain adjoining areas, and also in Calcutta city, during the years from 1917 to 1929 afforded an opportunity for a fresh investigation on the epidemiology of the disease. A phase of research was initiated in 1921 which has lasted up to the present time and during most of that period work has been especially directed towards the study of the sandfly as a possible vector of infection.

In 1921, an Inquiry on kala-azar was started at the Calcutta School of Tropical Medicine by L. E. Napier with the active collaboration of R. Knowles. Certain epidemiological observations were made regarding kala-azar in Calcutta which led these workers to believe that a limited entomological survey of a certain part of Calcutta would be likely to indicate the insect responsible for transmission, and mainly for this purpose R. O. A. Smith joined this Inquiry in 1924. This Inquiry continued on a varying scale up to 1938 with L. E. Napier and later R. O. A. Smith in charge. *Phlebotomus argentipes* was found abundantly in this area and these workers selected this insect first for investigation for this reason and also because its general distribution in India coincided with that of kala-azar.

In 1924 a special Kala-azar Commission which absorbed the already existing Kala-azar Inquiry of the Indian Research Fund Association under H. E. Shortt was constituted to carry out field and laboratory investigations in Assam. S. R. Christophers was appointed Director, with H. E. Shortt as protozoologist and P. J. Barraud as entomologist. The Commission was financed on a generous scale by the Indian Research Fund Association and was assisted by contributions from Local Governments concerned. In 1925, H. E. Shortt took over the Directorship and at varying periods A. C. Craighead, R. O. A. Smith, and K. V. Krishnan joined the Commission. C. S. Swaminath was a technical assistant during the period of investigation in Assam.

The first marked advance obtained was the finding of the development of typical herpetomonad forms in the fore-gut and mid-gut of the laboratory-bred *Phlebotomus argentipes* fed on parasite containing blood of kala-azar patients, by the Inquiry in Calcutta. Later this was confirmed by the workers in Assam.

A detailed account of the subsequent stages of the investigations on this species as a vector of kala-azar is embodied in the 1st and 2nd Reports of the Kala-azar Commission (*Indian Medical Research Memoirs*, Nos. 4 and 25) which cover the period up to 1931. Transmission to experimental animals by the bite of infected *Phlebotomus argentipes* had been obtained in a few instances but attempts to

transmit in this way to human volunteers had not been successful. Many efforts had been made to obtain this final proof and the reasons for failure were obscure. The large-scale field work of the Commission was abandoned in 1931 and the matter was left in this disappointing position although much epidemiological and experimental evidence which had been obtained left little doubt that *Phlebotomus argentipes* was the vector.

On the closure of the Commission the Inquiry at the Calcutta School of Tropical Medicine was at first strengthened and later work was continued on a diminishing scale and was eventually reduced to a level which would only ensure such degree of continuity of experience as would provide a basis for renewed work in event of a fresh opportunity arising.

In 1939, kala-azar had assumed epidemic proportions in Bihar and R. O. A. Smith was placed in charge of a new Inquiry in that Province. The sandfly was again studied and new methods of breeding, maintaining and feeding *Phlebotomus argentipes* were developed, and with their use a much greater success than formerly was obtained in the transmission of kala-azar to experimental animals, all hamsters used being infected. Unfortunately, on account of war conditions, it was found necessary to close this Inquiry in 1941. Some of the technical staff were combined with those of a Protozoological Inquiry in the Madras Presidency which had also to be terminated and a combined team was formed which was posted to Assam to continue kala-azar work under direction. At this time kala-azar was recrudescing in that Province and conditions existed in which it was possible to attempt again the transmission experiments to human volunteers under suitable control. The new technique devised by Smith for obtaining sandflies in a highly infective state was employed. The successful result of this work has been reported in the preceding paper by Swaminath, Shortt and Anderson.

Twenty years of patient investigation have gone to the forging of this final link in the evidence that *Phlebotomus argentipes* is the insect vector of human kala-azar in India, and the many workers who have taken part in the investigations at different stages and have contributed to the final solution of the problem are to be congratulated on this outcome of their work.

—EDITOR,

*Indian Journal of Medical Research*

## COMPARATIVE STUDY OF HUMAN IMMUNIZATION WITH TWO AND THREE DOSES OF TETANUS TOXOID

BY

D C LAHIRI

[Received for publication, August 25, 1942]

ALTHOUGH the active immunization of the fighting forces against tetanus toxoid has been widely practised since the outbreak of the war, the methods of immunization, as regards the number of doses and the interval between them, still vary. Ramon and Zoeller (1927) originally recommended a course of three injections of 1 c.c., 2 c.c. and 2 c.c., of tetanus toxoid at intervals of two or preferably three weeks. They also recommend a fourth dose of 2 c.c. a year later. This dosage and the intervals are adhered to in France. The United States Army practice is to inject three doses of 1 c.c. each subcutaneously at intervals of three weeks, and a stimulating dose of 1 c.c. at the end of the year (Bain, 1941). The method practised in immunizing British Forces 'is to inject two doses of 1 c.c. deeply into the subcutaneous tissues of the left arm at a time interval of six weeks' (Marvell and Parish, 1940). The British practice is based on the work of Boyd (1938). He immunized 30 men with two doses of 1 c.c. at six weeks' interval and 4 men with three doses at about three weeks' intervals. He concluded 'that inoculation with two doses of tetanus toxoid, given at an interval of six weeks or slightly over, has produced an antitoxin titre which is equal to that given by the three-dose method as advocated by Ramon and Zoeller (*loc cit*) and widely practised in France and elsewhere'. The great disparity in the size of the two groups of men immunized makes the evaluation of the results obtained difficult, and also because other workers prefer three-dose inoculation a fresh study was undertaken and the results are recorded in this paper.

### METHOD

A batch of toxoid which satisfied the antigenic requirements of both the British Therapeutic Substances Regulations and those laid down by the United States

Public Health Service, was selected for the experiment. A group of 46 members of the Institute was divided at random into two batches of 23 each. Those in group one were immunized with two doses of 1 c.c. each at six weeks' interval, and those in the other group were immunized with three doses of 1 c.c. each of the same toxoid, at an interval of three weeks between each dose. Samples of blood were collected from each of them four weeks after the last dose and the antitoxin content of their sera were determined at 0.2, 0.5 and 2.0 International unit levels.

### RESULT

The results are given in the Table below —

TABLE

*Antitoxic titre in men immunized with tetanus toxoid by two different methods*

Method of immunization	Number of samples	Antitoxic titre in International units per c.c.			
		<0.2	0.2 to 0.5	0.5 to 2.0	>2.0
Two doses of 1 c.c. at six weeks' interval	23	11	7	2	3
Three doses of 1 c.c. at three weeks' intervals	23	6	1	10	6

### DISCUSSION

In the three-dose group 16 men out of the total of 23 gave an antitoxin content of 0.5 unit and over, while only 5 men gave similar values in the two-dose group of the same size. While only 3 men of the two-dose group gave as high a value as 2 units and over, this figure was reached by 6 men in the three-dose group. As the titration was not carried out at a level higher than 2 units, it is, however, not possible to say what was the highest antitoxin content reached in the two groups. The point to note, however, is that almost half the number of the two-dose group (11 men out of 23) gave an antitoxin content less than 0.2 unit, while only 6 of the three-dose group gave such a low figure. These results show that three-dose inoculation at three weeks' intervals gives rise to a higher antitoxin content than two-dose inoculation at six weeks' interval.

## CONCLUSION

The active immunization of two groups of 23 men each with tetanus (formol) toxoid shows that three doses of 1 c c each injected at intervals of three weeks produced more antitoxin in man than two doses of 1 c c each injected at an interval of six weeks.

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## POST KALA-AZAR INFECTION OF THE SKIN BY *LEISHMANIA DONOVANI*

BY

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[Received for publication, July 23, 1942]

THE disease may be defined as leishmaniasis of the skin due to infection with *Leishmania donovani* in persons who have been cured of kala-azar after treatment or more rarely spontaneously or still more rarely in persons living in endemic areas of kala-azar but who do not give any definite history of a previous attack of the disease. Very rarely the disease has been observed in persons at a period when they were still suffering from internal leishmaniasis or not completely cured of it. Further, except in extremely rare cases, the disease, so far observed, is non-ulcerating.

Some controversy has arisen about the name of the condition and various synonyms have been suggested. The original name 'dermal leishmanoid' adopted by the discoverer Brahmachari (1922) has been used by many writers. Thus, Shortt has used the same name in all his writings, and in the last Report of the Kala-azar Commission (Shortt, 1932), the same nomenclature has been used. In the index to the *Tropical Diseases Bulletin*, the original name was adopted for several years after its discovery.

Knowles (1928), in criticizing the various names adopted, wrote as follows, as Editor, *Indian Medical Gazette*. 'The name (dermal leishmanoid) was not altogether satisfactory. The suffix "oid" denotes "resemblance to the thing indicated by the preceding element of the compound", to quote from a well-known medical dictionary. Syphiloid suggests a lesion resembling syphilis but not necessarily caused by *Treponema pallidum*. Now, the most striking thing about these lesions was their lack of resemblance to any previously described *leishmania* lesion, except possibly the lesions described by Thomson and Balfour (1909) for which they suggested the name "leishman nodules". On the other hand, working on the same analogy leishmanoid would have been a more suggestive name



The whole series of changes are due to a widespread infection of the skin by *leishmania*, the lesions are mostly confined to the skin proper. The term "dermal leishmaniasis" is, therefore, descriptive. But oriental sore is very frequently referred to as dermal leishmaniasis, so to avoid all risk of confusion, it was necessary to qualify further the condition under discussion. Post kala-azar dermal leishmaniasis is a clumsy name, but it defines the condition, the alternative name post-generalized dermal leishmaniasis is certainly worse.

Brahmachari (1929) pointed out that the name leishmanide suggested by the Editor, *Indian Medical Gazette*, was not free from objection for the following reasons: a name ending in *ide* frequently gives an impression of an *amide*, such as tryparsamide, and confusion may arise in the mind of the reader as to whether leishmanide is the name of a specific for leishmaniasis or the name of a cutaneous manifestation of infection with *leishmania*. He was, therefore, inclined to think that on the whole 'dermal leishmanoid' was the most appropriate, if not the most convenient, for the disease. He stated that the name had the sanction of usage, and most observers recognized the disease under the name.

What then should be the proper nomenclature of the disease? The term 'dermal leishmanoid' has had the usage of time. Why then change it? One cannot agree 'oid' always denotes 'resemblance to the thing indicating the preceding element of the compound'. Thus, varioloid does not mean a disease resembling variola. It means variola modified by vaccination or by a previous attack of smallpox. So 'dermal leishmanoid' means infection with *Leishmania donovani* modified by a previous attack of kala-azar cured after treatment or more rarely spontaneously. But as varioloid means a re-infection, the two names are not quite comparable.

I agree with Knowles that the name post kala-azar dermal leishmaniasis is a clumsy one.

Megaw (1922) suggested the name 'Brahmachari's dermal leishmaniasis' after the name of the discoverer. Many diseases have been named after the discoverers, such as, Basedow's disease, Osler's disease, Oppenheim's disease, etc. I would suggest that the disease may be henceforth known as 'dermal leishmanoid' or 'Brahmachari's disease' in analogy to other names such as, exophthalmic goitre (= Basedow's disease or Graves' disease).

That a disease primarily a very fatal internal malady should as a result of specific treatment be converted into an affection of the skin harmless so far as the life of the affected individual is concerned must be a unique phenomenon in immunology.

In kala-azar the parasites may be carried to the different parts of the body by cells belonging to the reticulo-endothelial system and also by other leucocytes. Those among them which contain a large number of leishmania, viz the hyaline mononuclear leucocytes (Knowles, 1920), the clasmatoocytes (Cash and Hu, 1927), the monocytes or histiocytes are probably *leishmania*-protective and those that contain the least number are probably *leishmania*-destructive. Among the *leishmania*-containing cells in 'dermal leishmanoid' are the dermal melanophores, the

large extravascular cells containing small nuclei and a large amount of cytoplasm, the cells constituting the walls of the newly formed blood vessels in the granulation tissue, cells with branching processes in the deeper layer of the dermis, the monocytes, the neutrophils and the giant cells. Besides, some of the *leishmania* are found in extravascular spaces. Some of the above cells are undoubtedly *leishmania*-protective and help in the growth of the *leishmania* in the skin. In 'dermal leishmanoid' the following may be considered to happen (1) preponderance of *leishmania*-protective cells in the skin, (2) multiplication and growth of the parasites inside them or of those that lie in extravascular spaces. When a sufficient number of *leishmania* have grown in the skin they give rise to the appearance of skin lesions constituting a definite clinical entity ('dermal leishmanoid')

Under ordinary normal conditions or in kala-azar before commencement of any specific treatment, these protective cells are present in small numbers in the skin and therefore in such cases there is no tendency on the part of the *leishmania* to concentrate in the skin as long as no specific treatment is begun.

In the depigmentation of the skin in 'dermal leishmanoid' there are destruction of the pigment-carrying cells of the epidermis and diminution of their pigment, spreading sometimes over extensive areas. These areas of depigmentation may remain for indefinite periods without the presence of any nodules or papules. In such cases of depigmentation frequently no *leishmania* can be seen in a smear preparation but they can sometimes be demonstrated by cultural methods.

One notable fact about 'dermal leishmanoid' is that the extent of lesion in the skin whether nodule or papule is not always proportional to the number of *leishmania* present in them.

It is a striking phenomenon that while darkening or hyperpigmentation of the skin is characteristic of kala-azar as the name of the disease implies, the reverse condition, i.e. depigmentation, is more frequently characteristic of 'dermal leishmanoid'. Why there should be two opposite features in the skin in diseases due to the same parasite is difficult to explain. An explanation is offered here. In 'dermal leishmanoid', the pigment cells are directly attacked by *leishmania* as has been found to be the case and the superficial layers of the skin are more or less destroyed and as a result of this the number of pigment cells diminish and the pigment-carrying property of the pigment cells is lost, due probably to the action of the metabolic products of *leishmania* inside them, while in kala-azar hyperpigmentation may be indicative of the action of the toxin of the *leishmania* upon the suprarenal glands as is indicated by tachycardia, low blood pressure and pulsation of the veins in the neck. Any hyperpigmentation or other signs of suprarenal insufficiency that might result from the action of the toxin of *leishmania* upon the suprarenal glands in 'dermal leishmanoid' may be considered to be prevented either by being attenuated during treatment or by the antibodies present in a cured case of kala-azar, the general symptoms of kala-azar, such as fever, wasting, anaemia, emaciation, etc., followed by high mortality, are completely absent in 'dermal leishmanoid' even in cases in which the lesions in the skin are most extensive.

## EXPLANATION OF PLATE XII

### *Section of skin in different stages of the disease*

- Fig 1 Photomicrograph of a section showing perivascular round cell infiltration just below the epidermis
- „ 2 Photomicrograph of a section showing extension of perivascular round cell infiltration to surrounding tissues
- „ 3 Photomicrograph of a section showing thinning of epidermis before any nodule or papule formation
- „ 4 Photomicrograph of a section showing extravascular *leishmania* in the deeper layer of granulation tissue
- „ 5 Photomicrograph of a section showing *leishmania*-laden pigment-carrying cells in superficial layer of dermis
- „ 6 Photomicrograph of a section showing extravascular *leishmania*-laden cells
- „ 7 Photomicrograph of a section showing *leishmania* inside cells with branching processes in the deeper layer of the dermis
- „ 8 Photomicrograph of a section showing marked involvement of epidermis which is very much thinned out
- „ 9 Photomicrograph of a section showing *leishmania*-laden endothelial cells in the walls of capillaries
- „ 10 Photomicrograph of a section showing cedema of the tissue just below epidermis
- „ 11 Photomicrograph of a smear showing *leishmania* from a cut nodule
- „ 12 Drawing from a section showing irritative hypertrophy with epithelial pearls in a case of ulcerating 'dermal leishmanoid'
- „ 13 Drawing from a section showing certain cells containing *leishmania* from a smear of a cut nodule (Giemsa) Note a giant cell containing *leishmania* There are some monocytes containing *leishmania*

PLATE XII.



Fig 1



Fig 2



Fig 3



Fig 4.



Fig 5

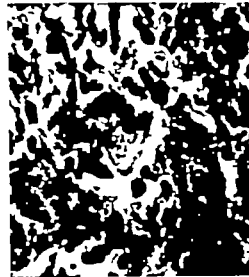


Fig 6

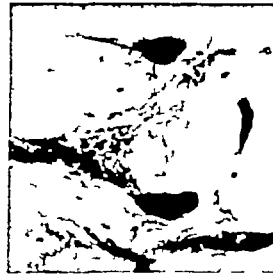


Fig 7

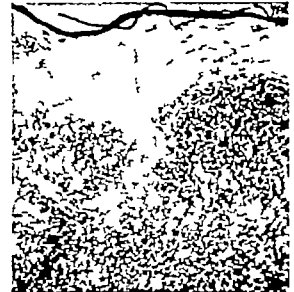


Fig 8

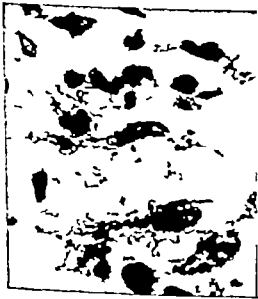


Fig 9

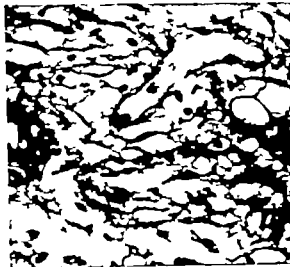


Fig 10

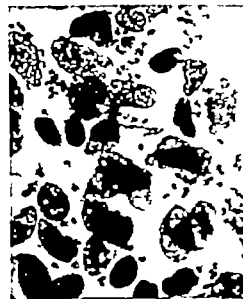


Fig 11

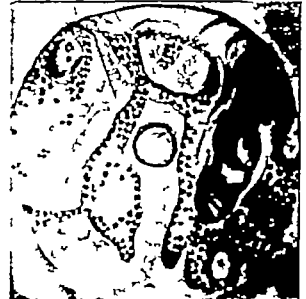
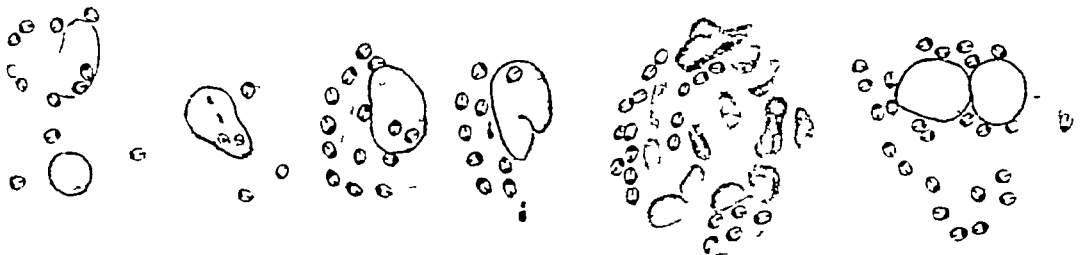


Fig 12



intramuscular treatment they would be easily and quickly attacked by antimony and the chances of 'dermal leishmanoid' developing in them in this way be reduced to a minimum

The disease appears to be an infective granuloma involving the superficial areas of the dermis and the pigmentary layer of the epidermis. At an early stage there is a perivascular infiltration of cells in the dermis just below the epidermis. As the disease progresses there is increase of this infiltration which extends into the surrounding parts of the skin.

In some cases the epidermis is attacked more than the dermis and as a result of this, thinning of the epidermis with depigmentation occurs before any papule or nodule formation takes place or at any rate before they are visible to the naked eye. In this type of the disease extensive well-marked areas of the depigmentation are seen in the skin in the different parts of the body and such a condition may remain for indefinite periods without any papule or nodule formation.

In other cases papules appear in the skin either in the depigmented patches or in areas which do not apparently show any depigmentation. On examination of the papules with a hand-lens the papillæ of the skin may appear to be more prominent than normal but in most cases there is no true hypertrophy. An apparent hypertrophy is really due to the tissues of the papillæ being involved by an excessive amount of round cells attacking the papillary layers of the dermis. Sooner or later the epidermis begins to thin out but except in extremely rare cases some portion of the epidermis remains intact which is contrary to what is found in oriental sore.

Some observers have stated that here and there in the large capillaries the endothelium is hypertrophied and the wall thickened and in some cases there is a very marked thickening of the wall of the capillaries almost leading to their obliteration. In view of the fact that ulceration is extremely rare in any part of the affected portion of the skin, whether it is a depigmented patch or a papule or a nodule, the circulation in the skin cannot be so much diminished by the thickening of the blood vessels as to cause the death of the affected tissues which might lead to ulceration.

Very rarely, however, the epithelial lining over the granulation tissue while thinning out at one part may ulcerate at another. In such cases the papillary layer of the skin may be seen to throw out processes into the granulation tissue underneath (irritative hypertrophy). The ulcerated area may be covered with coagulated lymph with some leucocytes entangled in it. Remains of such apparently hypertrophied papillæ may also be seen in the granulation tissue in the ulcerated area. Here and there small epithelial pearls may be seen in some of them.

In some rare cases there is a hyperpigmentation of the skin, and there is a larger amount of pigment in the pigment cells of the epidermis in the affected part than in the surrounding parts.

In certain cases, one of the earliest changes in the skin is congestion of the superficial blood vessels as shown by erythema. Histologically, this is shown by

the dilatation of the blood vessels in the superficial layer of the skin with some amount of perivascular infiltration of the dermis

Some observers consider that erythema first manifests itself before any depigmentation occurs. But this non-involvement of the epidermis is rare and generally in practice one finds more or less an early involvement of the pigment layers of the epidermis and many patients come for treatment of the depigmented patches which, they state, showed themselves before the appearance of the erythema.

More frequently there is secondary erythema in the skin with papule formation. In these there is depigmentation of the skin with thinning of the layers of the epidermis over the erythematous areas and infiltration of round cells extending upwards towards the epidermis and downwards into the dermis. There is also the evidence of newly formed blood vessels below the epidermis. Along with this there is dilatation of blood vessels below the epidermis.

In the case of well-formed papules and of nodules, the following changes are noticeable in the structure of the skin. Just below the epidermis, there is a layer of well-marked round-celled infiltration the area of which varies in thickness. In the superficial layer of this granulation tissue just below the epidermis newly formed blood vessels are seen.

Along with this, *leishmania*-laden cells situated close under the epidermal layer begin to show themselves. Many of these *leishmania*-laden cells contain pigment granules others do not. As the papules increase in size, pigment-carrying cells containing *leishmania* are seen in the superficial layers of the dermis. These cells are probably dermal chromatophores infected with *leishmania*.

The *leishmania*-laden pigment cells in the granulation tissue are frequently swollen and do not always appear to be in connection with blood vessels. In addition to these cells *leishmania* may be found inside extravascular cells which contain no pigment. In other cases they are present inside endothelial cells in the walls of the capillaries.

In the deeper layer of the granulation tissue, *leishmania* may be found inside extravascular cells. In the deepest layer of the dermis where the granulation tissue is merging into the areolar tissue a few *leishmania* are seen inside cells with branching processes. In some cases *leishmania* are found free in the granulation tissue of the affected part of the skin.

In those cases in which papules or nodules appear in depigmented patches the tissue changes are characterized by more or less marked involvement of the epidermis which may sometimes be very extensive.

Generally speaking, it may be stated that there is some amount of involvement of the epidermis whenever there is presence of well-marked papule or nodule formation. This is greatest in those places where there is presence of depigmentation of the skin along with papule or nodule formation and less marked in those cases where there is no depigmentation. But even in those cases where there is no naked-eye appearance of depigmentation some amount of involvement of the epidermis is frequently noticeable.

In many cases there is oedema of the dermis just below the epidermis and where there is well-marked round-celled infiltration the white elastic fibres are destroyed along with the normal connective tissue by the round-celled infiltration.

Contrary to what occurs in other affections of the skin the hair follicles are not involved in the infective process, though in course of time the round cell infiltration may extend to them as well as to the sweat glands.

The *leishmania* are best seen in smears from a cut papule or nodule.

It has been noted that the number of *leishmania* seen in a section of the skin is not always proportionate to the extent of the lesion present. Thus, the author noted that in a case a few scanty *leishmania* were present inside the papules in 1926, while similarly scanty *leishmania* were present in 1933 in well-marked nodules. In other words, the amount of tissue changes is not proportional to the number of *leishmania* present.

Roughly speaking, it may be stated that the proportion of *leishmania* present in a lesion is in the following order —

(1) Erythematous patch	No <i>L D</i> bodies present
(2) Depigmented patch	None or very few present
(3) Papules	<i>L D</i> bodies present
(4) Nodules	<i>L D</i> bodies present

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## VITAMIN C IN GERMINATING GRAINS

BY

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[Received for publication, September 26, 1942]

As long ago as 1782 the use of germinated pulses as an anti-scorbutic for the British Navy was suggested by Young (Medical Research Council, 1932). It was not however, until the present century that Furst (1912), working in Hölst's laboratory in Oslo, showed that cereals and pulses develop anti-scorbutic properties after sprouting. This was confirmed for pulses by Chick and Hume (1917) and Chick and Delf (1919), who recommended germinated pulses as a source of vitamin C in army rations in the absence of fresh fruit and vegetables. Wiltshire (1918) succeeded in curing several cases of mild scurvy by including germinated haricot beans in the diet.

Chick and Delf (*loc cit*) and Wats and Evles (1932) made a quantitative study of the anti-scorbutic value of germinating seeds employing the biological method which, though it gives reliable results is time-consuming and involves the use of considerable numbers of experimental animals. The chemical method originally described by Tillmanns Hirsch and Hirsch (1932) is now universally used for estimating the vitamin C potency of foods. The chemical method, besides being quick and accurate, gives results which are comparable with those obtained by the biological method.

In China, various sprouted seeds have been tested for their vitamin C content by Chu and Read (1938) who employed the chemical method. The vitamin C

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content of some Indian pulses and cereals on germination has been determined by Guha and Ghosh (1934), Ahmad (1935), Rudra (1938) and Ahmad and Muthanna (1940)

Germinated pulses are a common article of diet in certain parts of India, the pulse which is most commonly consumed in this state being Bengal gram (*Cicer arretinum*). Sprouted Bengal gram is also used in child-welfare centres as an inexpensive source of vitamin C. Sprouted pulses have been used in the prevention of scurvy in a famine area (Nicol, 1941, Khan, 1942). In war time the study of available sources of vitamin C becomes of importance. It was therefore felt that a re-investigation of the amount of vitamin C present in various common pulses, in relation to the period of soaking and sprouting, etc., would be of interest and value.

### EXPERIMENTAL

Whole seeds were used for germination. The seeds were soaked for 12 hours in tap-water and then spread between two layers of cloth, which was kept moist by sprinkling water now and then. Unless otherwise stated, the soaking and germination took place at Coonoor room temperature in the warm weather (21°C to 25°C) and in diffused daylight. For the estimation of vitamin C, the 2,6-dichlorophenol-indophenol-titration method was employed. Two to five grammes of the material were taken for each estimation and soaked in 10 cc of 8 per cent trichloroacetic acid in order to minimize the oxidase activity. They were then extracted by the method of Bessey and King (1933) employing 8 per cent trichloroacetic acid. Three extractions were found to remove almost all the vitamin C and the extracts were stable for 1 to 2 hours. The extracts were titrated rapidly against a measured quantity (0.1 cc to 0.25 cc) of the standard dye solution which was standardized against pure ascorbic acid. In the case of each batch of germinated grains, the dry weight was determined by drying in the steam-oven and the vitamin C content expressed on the dry weight basis for facilitating comparison of the results.

*Vitamin C content of dry seeds*—In order to follow the production of vitamin C on germination, it was felt advisable to test the dry seeds. It is usually taken for granted that dried pulses are devoid of vitamin C or contain only traces. Most of the dry pulses, and the three cereals tested, gave low or negligible values in terms of the chemical test. A few samples of Bengal gram purchased locally were, however, found to give figures ranging from 8 mg/100 g to 19 mg/100 g. This was somewhat surprising and we felt that it might be due to some abnormality in the local samples. Accordingly, a series of ordinary bazaar samples were obtained from various parts of the country, 22 in all being tested. All samples gave values within approximately the same range. Dry green gram was also found to contain some vitamin C. Low values were given by other grains in the dry state.

The results of the tests carried out on the dry grains are shown in Table I —

TABLE I  
Vitamin C content of dry seeds

Pulses	Sample number	Obtained from	Mg vitamin C, per cent	Nitroprusside reaction as Lovibond units per gramme
Bengal gram ( <i>Cicer arietinum</i> )	1	Coonoor market	13.26	11.40
"	2	"	13.36	11.40
"	3	"	8.42	10.50
"	4	"	12.50	9.90
"	5	"	12.75	6.00
"	6	"	18.70	4.80
"	7	Coimbatore	20.00	10.80
"	8	Ranipet, Madras	15.60	12.00
"	9	"	7.90	
"	10	"	10.60	
"	11	"	6.80	6.00
"	12	Bangalore	10.00	
"	13	"	6.00	
"	14	Madras City	14.30	7.20
"	15	"	10.00	
"	16	"	12.50	
"	17	"	6.70	5.40
"	18	Lahore	14.00	11.40
"	19	"	11.20	
"	20	"	13.40	
"	21	"	16.90	13.80
"	22	"	14.50	
Green gram ( <i>Phaseolus radiatus</i> )	1		8.67	
"	2		9.95	6.00
"	3		8.90	6.00

TABLE I—concl'd.

Pulses	Sample number	Obtained from	Mg vitamin C, per cent	Nitroprusside reaction as Lovibond units per gramme
Field beans ( <i>Dolichos lablab</i> )			1 20	
Peas ( <i>Pisum sativum</i> )	1		3 20	18 00
„	2		5 80	25 00
Cow-pea ( <i>Vigna catuag</i> )	1		2 04	
„	2		1 53	6 00
„	3		2 30	9 60
Soya bean ( <i>Glycine hispida</i> )			1 10	Trace
Khesari ( <i>Lathyrus sativus</i> )		Central Provinces	4 50	
Cereals				
Rice ( <i>Oryza sativa</i> )			Trace	Nil
Jowar ( <i>Sorghum vulgare</i> )			„	„
Wheat ( <i>Triticum vulgare</i> )			„	„

It was thought that the indophenol titre given by dry Bengal gram might be due to the presence of some other reducing substances, e.g. cystine or glutathione. On testing with nitroprusside the various seeds gave a positive reaction. A quantitative study of the reaction was made by comparing the red colour formed on the addition of sodium nitroprusside and ammonia in a Lovibond tintometer and the intensity of the colour was expressed as red Lovibond units per gramme of the material. It will be seen from Table I that the intensity of the nitroprusside reaction did not run parallel with the concentration of vitamin C in the various dry seeds. Peas, which gave the strongest nitroprusside reaction, gave a lower indophenol titre than any of the samples of Bengal gram. Further, the indophenol titrations were completed within 10 to 15 seconds and substances other than vitamin C will not reduce the dye in so short a period (Millikan, 1935). Additional confirmation was obtained by treating the extract with Norit, which converts ascorbic acid into its dehydro form (Fox and Levy, 1936), which does not reduce the dye. The difference between the titre values before and after Norit treatment will indicate the amount of ascorbic acid present in the extract. Experiments with Norit provided additional evidence that the dye-reducing substance was in fact

vitamin C Finally the results of the chemical tests on Bengal gram were confirmed by biological experiments, the details of which are given in another paper (Bhagvat and Rao, 1942)

*Synthesis of vitamin C during germination*—In these experiments an attempt was made to adjust the conditions of germination so that they approximated to those commonly adopted in Indian homes. The seeds are usually soaked overnight and then spread on wet cloth. In order to study the effect of soaking green gram was soaked for different periods and its vitamin C content determined. The other pulses and cereals were tested for vitamin C after soaking for 12 hours. The results are incorporated in Table II —

TABLE II

*The effect of soaking on the vitamin C content of various pulses and cereals*  
(Vitamin C, mg per 100 g of dried material)

Period of soaking, hours	PEAS		Green gram (mean of 4 samples)	BENGAL GRAM		Cow pea (mean of 3 samples)	Jowar (one sample)	Wheat (one sample)
	Sample I	Sample II		Sample I	Sample II			
Dry	3.2	5.8	9.0	13.2	20.0	1.53	Trace	Trace
4			10.7					
8			14.4					
12	4.3	6.7	14.3	15.3	21.4	2.30	1.8	1.8
16			14.8					
24			16.2		22.7			

Very little vitamin C is formed on merely soaking the seeds in water from 4 to 12 hours. In the case of green gram some increase in vitamin C content was observed after 24 hours' soaking, which was due to the fact that germination had started by the end of that period. In all subsequent experiments the seeds were soaked in water for 12 hours prior to germination. This conforms with the common domestic practice of soaking the seeds overnight.

Next the synthesis of vitamin C during germination was studied. In the case of Bengal gram, cow-pea and green gram, experiments were conducted at two different temperatures. The results of various experiments are presented in Table III —

TABLE III  
Vitamin C content of germinating grains

(Vitamin C, mg per 100 g of dried material)

Grains — Period of germination in hours	Khesari dhal.	Bengal gram		Green gram		Cow pea		Peas		Soya bean	Wheat	Jowar
		37°	R T	37°	R T	37°	R T	37°	R T			
5½		19.4	17.1	30.5	18.3	8.4	3.1					
24	16.2	27.5	26.5	35.5	38.3	31.7	32.5	12.1	15.1	5.2	5.1	2.1
29			32.2	40.8	54.3	36.1	43.3					3.0
48	16.7			40.8	53.3	34.9	47.6	20.3	28.3	10.8	8.8	4.6
53½		26.6	34.5	41.0	53.9	33.4	48.5				8.8	
72	16.2	27.9		42.0	54.8	33.0	48.5	25.3	31.0		8.8	8.9
96	16.6		31.0		54.8	34.0					8.8	

R T = Room temperature in the warm weather, i.e. about 21°C to 25°C

The following facts are evident from Table III. Pulses and cereals rapidly synthesize vitamin C immediately after the initiation of germination. In the case of Bengal gram, green gram, cow-pea and wheat, the maximum amount of vitamin C was synthesized within 48 hours, after which it remained constant for 4 days. The germination was discontinued after 4 days, as in practice pulses are germinated only for 24 to 48 hours before they are consumed. Germinated pulses are a better source of vitamin C than germinated cereals. Of the pulses studied green gram synthesized the largest amount for vitamin C and soya bean the smallest. The vitamin C content for green gram increased 7 to 8 times on germination (*cf* Guha and Ghosh, *loc cit*), while with cow-pea the increase was 20 to 25 times. At the higher temperature, germination, as judged by the length of the sprout, took place more rapidly than at the lower temperature, but the amount of vitamin C synthesized was greater at the lower temperature. This is in agreement with the findings of Virtanen and Eerola (1936). The samples sprouted at 37°C were kept in the incubator, and it was felt possible that the lower values given by these might have been due to the exclusion of light. Experiments to control this point, in which samples were kept with equal exposure to light outside and inside the incubator (the latter with its wooden door open so that light penetrated through the glass door), showed that the difference in light was not responsible for the result.

The figures shown in Table III represent results obtained with typical samples. Actually the amount of vitamin C present after 24 hours' germination in different samples of the same pulse showed considerable variation. Thus, with Bengal gram, a variation of 19 mg/100 g to 27 mg/100 g, with green gram, 39 mg/100 g to 63 mg/100 g, and with cow-pea, 36 mg/100 g to 40 mg/100 g was observed. With all samples, however, the trend was the same for each individual kind of pulse.

It was felt to be of interest to see how much of the vitamin is present in its dehydro form, which, though biologically active, does not react with the dye. Trichloroacetic acid extracts of Bengal gram, green gram and cow-pea were subjected to  $H_2S$  treatment.  $H_2S$  was removed by a current of  $CO_2$ , and the vitamin C was titrated against the dye. The amount of vitamin C found was the same before and after  $H_2S$  treatment, indicating the absence of the dehydro form in germinating pulses. Thus, the findings of Lee and Read (1936) and Rudra (*loc cit*) that ascorbic acid in germinated grains is mostly present in the form of the reversibly oxidized dehydro ascorbic acid have not been substantiated. Harris and Olliver (1942) state that 'the quantity of dehydro ascorbic acid normally found in fruits and vegetables is so small as to be of little or no practical significance'.

*Distribution of vitamin C in germinating pulses*—Vitamin C is rapidly formed in all sprouting seeds (Sherman and Smith, 1931, Pfankusch, 1934) and it is probably present in high concentration in rapidly growing stems, root tips, green leaves, seeds and pods. Gluck (1936, 1937), employing the micro-Linderstrom-Lang technique, made a detailed study of the distribution of vitamin C in various parts of the germinating barley grain. He found an increase in the concentration of vitamin C in those parts of the seed where chlorophyll was present and photosynthesis was active.

Lee (1936) and Lee and Read (*loc cit*) found that the sprouts from germinated peas and soya beans showed a fairly high concentration of the vitamin. The distribution of the vitamin in common Indian pulses during germination has not been studied. In the Ajmer famine of 1940, in which germinated Bengal gram was distributed on a wide scale, it was observed that the people often picked off the shoot and threw it away before eating the pulse (Aykroyd, private communication). It was not known at that time how much vitamin C was being lost in this way. Accordingly, the distribution of vitamin C in the cotyledon and sprout was studied. The seeds were germinated for different lengths of time, the cotyledons separated from the sprouts and their vitamin C content estimated. The results of experiments on single samples of each pulse are shown in Table IV. Other samples, tested at varying periods after germination, gave similar results --

TABLE IV

*Distribution of vitamin C in germinating seeds*

(Percentage of vitamin present in entire germinating grain)

Period of germination, hours	Bengal gram		Green gram		Cow pea		Field beans		Wheat	
	C	S	C	S	C	S	C	S	C	S
24	80.6	19.4	63.2	36.8	71.4	28.7	68.8	31.2		
48	61.7	38.3	57.1	42.9	49.3	50.7	52.6	47.4		
72	52.6	47.3	50.0	50.0	50.0	50.0	43.1	56.9		
96	44.0	56.0	43.2	56.8					27.5	72.5
120			31.6	68.4						

C = Cotyledon

S = Sprout

The vitamin is apparently formed in the cotyledon and is then transferred to the rapidly growing sprout, where the amount present goes on increasing as germination progresses. Thus, a considerable amount of the vitamin C is lost if the sprouts are thrown away.

*Effect of cooking and drying on the vitamin C content of germinating pulses* — A great deal of work has been carried out on the effect of cooking, canning and drying of different vegetables and fruits on their vitamin C content, but germinated pulses

have not received much attention. In India germinated pulses form a common constituent of the diet. They are sometimes consumed raw, but they are also commonly taken in the cooked state. A study of the effect of cooking is therefore of practical interest. The various pulses were germinated for 24 hours and then cooked in water for different periods, the conditions of cooking etc. being kept constant in all the experiments. Vitamin C was determined titrimetrically and the percentage loss was calculated from the amount of the vitamin remaining both in the cooked material and the cooking water (Table V) —

TABLE V

*Effect of cooking on the vitamin C content of germinated pulses*  
(Percentage loss of vitamin C)

Period of cooking, minutes	BENGAL GRAM (1 SAMPLE)		Green gram (mean of 2 samples)	Cow pea (mean of 3 samples)	Field beans (1 sample)
	Germinated	Dry			
10	12.0	7.0	30.3	32.0	25.2
20	19.1	30.0	42.7	49.8	28.0
40	52.1	41.0	50.0	75.5	81.9
60	65.9	53.0	52.8	87.7	87.1

Germinated pulses, like most vegetables and fruits show a considerable loss of vitamin C on cooking. Bengal gram and green gram lost less vitamin C on cooking than cow-pea and field beans. The sample of dry Bengal gram lost less vitamin than the germinated sample. All the pulses were fully cooked in about 20 to 40 minutes. Thus in order to minimize loss of vitamin C it is advisable not to prolong cooking beyond half an hour. Cooking the germinated pulses in a slightly acid medium might help to reduce the loss. The maximum anti-scorbutic effect will of course be obtained if sprouted pulses are taken raw.

Next the effect of drying on vitamin C content was investigated. Dried vegetables are now being used for military purposes and if germinated pulses could be dried and stored without serious loss of vitamin C they might be of practical value as an anti-scorbutic. The two common pulses, Bengal gram and green gram, were used for these experiments. One sample of each was soaked in water for 24 hours, dried on a hot plate in a current of air for 40 to 60 minutes, and then preserved in tins at 37°C. Various methods of drying were tried, and the



hot-plate method was resorted to as it brings about quick drying of the material. Other samples were germinated for 24 hours prior to drying. The effect of blanching the pulses before they were dried, was tried in view of the findings of Beckley and Noltey (1941) that blanching is necessary in order to ensure minimum loss of vitamin C in potato and cauliflower during dehydration. Results are summarized in Table VI. The values obtained for samples of dehydrated germinated pulses prepared by a firm now engaged in the manufacture of dehydrated vegetables are also included —

TABLE VI

*The effect of drying on the stability of vitamin C in germinated pulses*

Pulses	Treatment prior to drying	Kept at 37°C for	Mg vitamin C, per cent	Percentage loss of vitamin C
		Days		
Green gram	Soaked for 24 hours		14.8	26.0
		7	8.5	57.5
		15	6.7	66.5
		30	2.8	86.0
Green gram	Germinated for 24 hours		32.9	52.4
		10	16.1	76.7
		17	10.7	84.5
		32	5.0	92.1
Green gram	Germinated for 24 hours and blanched for 3 minutes		51.3	18.7
		8	24.5	61.2
		26	14.0	79.4
		52	9.4	85.0
Bengal gram	Soaked for 24 hours		16.0	25.0
		43	5.6	74.0
Bengal gram	Soaked for 24 hours and blanched for 3 minutes		16.0	25.0
		43	5.6	74.0

TABLE VI—*concl'd*

Pulses	Treatment prior to drying	Kept at 37°C for	Mg vitamin C, per cent	Percentage loss of vitamin C
		Days		
Bengal gram	Germinated for 24 hours		17.4	11.2
		8	10.9	44.4
		23	7.4	62.2
		52	3.4	83.0
Bengal gram	Not soaked or germinated		20.0	Nil
		90	20.0	"
<i>Samples supplied by a commercial firm —</i>				
Horse gram	Germinated		Trace	
Cow pea	"		1.9	
Green gram	"		6.3	
Bengal gram	"		5.7	

The pulses lost from 11 to 26 per cent of the vitamin C originally present on drying. Further, they showed a progressive loss of the vitamin when the dried samples were kept at 37°C. 'Blanching' prior to drying did not seem to minimize the loss of vitamin C. A higher percentage of vitamin C in sprouted Bengal gram survived drying than was the case with sprouted green gram. Dry samples of ordinary Bengal gram as obtained from the market did not show any loss of vitamin C even on storage at 37°C for months—a finding of practical interest. Soaking of the sample in water, however, altered the stability of vitamin C in the gram and fairly rapid loss took place on storage. The mechanism in dry Bengal gram which protects the vitamin from destruction is a matter for further investigation.

These investigations showed that the use of dried germinated pulses as an anti-scorbutic cannot be recommended, the loss of vitamin C on storage is too rapid. The ease with which ordinary dry pulses can be germinated renders their previous germination and drying unnecessary.

Sprouted green gram gave higher vitamin C values than other pulses after germination and should be given preference when sprouted pulses are being used for anti-scorbutic purposes. In the raw state 2 oz. to 3 oz. in terms of the dry pulse (4 oz. to 6 oz. of the sprouted seeds) would supply about 25 mg. of vitamin C.

Cooking for 20 to 40 minutes would reduce vitamin C content by approximately half, but even this amount of the vitamin would be a valuable addition to a diet deficient in fresh fruits and vegetables

### SUMMARY

1 A study has been made of the effect of germination, cooking and drying on the vitamin C content of certain pulses and cereals

2 In the dry state, most of the grains contained small or negligible amounts of vitamin C Bengal gram, however, was found to contain from 6 mg to 20 mg, and green gram 8 mg to 10 mg per cent

3 Soaking had little effect on vitamin C content

4 Vitamin C is rapidly formed when germination begins The concentration reaches its maximum in 30 to 48 hours, after which it remains constant for 3 to 4 days Green gram gave the highest vitamin C titre after germination

5 Vitamin C is found both in the cotyledon and the sprout, the percentage present in the latter increasing, and that present in the former decreasing, as germination proceeds

6 Considerable loss of vitamin C takes place when germinated pulses are cooked The drying of germinated pulses also leads to loss, and the vitamin C which remains after drying is not stable on storage

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## VITAMIN C CONTENT OF DRY BENGAL GRAM (*CICER ARIETINUM*)

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[ Received for publication, September 20, 1942 ]

In the previous paper (Bhagvat and Rao, 1942) it was shown that various samples of dry unsprouted Bengal gram obtained from different parts of India contained significant amounts of vitamin C, as determined by the chemical method of Tillmanns, Hirsch and Hirsch (1932). The validity of this observation was confirmed by various chemical procedures. Since, however, it conflicted with the findings of earlier workers it was thought advisable to carry out biological tests, which provide a final court of appeal. Wats and White (1931) reported that the inclusion of Bengal gram in a basal diet at a 30 per cent level afforded guinea-pigs no protection from scurvy.

Young guinea-pigs weighing from 250 g to 350 g were used as experimental animals. They were kept on a stock diet rich in vitamin C for a week and then put on the experimental diets.

Two different samples of Bengal gram were tested, using two batches of animals. The first (sample A), obtained from the local bazaar gave a vitamin C titration value of 12.75 mg/100 g, and the second (sample B) obtained from the Government Agricultural College, Coimbatore (strain 445), gave a value of 20.00 mg/100 g. The test samples were coarsely powdered daily, in order to avoid

any loss of vitamin C which might occur in powdered samples on keeping The composition of the different diets was as follows —

Diets	Basal diet, parts	Experimental diet I (Bengal gram sample A), parts	Experimental diet II (Bengal gram sample B), parts
Crushed oats	31 0	14 0	20 0
Atta	31 0	14 0	20 0
Skimmed milk powder	30 0	14 0	20 0
Gingelly oil	5 0	5 0	5 0
Shark liver oil	1 0	1 0	1 0
NaCl	1 0	1 0	1 0
Yeast	1 0	1 0	1 0
Bengal gram		50 0	32 0
	100 0	100 0	100 0

The diets were moistened with water and given in the form of balls The food residue left over was weighed and the amount of vitamin C obtained by the animals on experimental diets I and II calculated The animals under experiment were grouped as follows —

- GROUP I 5 animals as negative controls, i.e. receiving the basal diet only
- GROUP II 4 animals as positive controls, i.e. receiving the basal diet plus 5 mg of pure ascorbic acid daily
- GROUP III 6 animals on experimental diet I
- GROUP IV 5 animals on experimental diet II

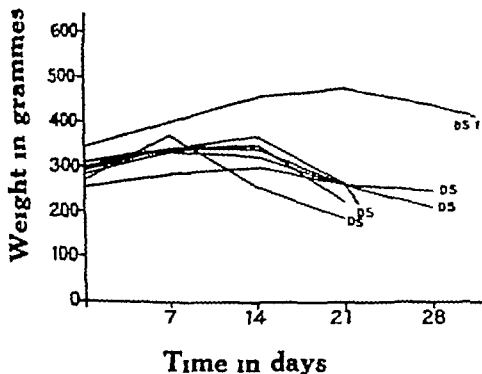
The animals were weighed weekly. The growth of the animals in the different groups is shown diagrammatically in Graph 1 —

GRAPH 1

*Weight curves of guinea-pigs receiving diets supplemented by ascorbic acid and Bengal gram respectively*

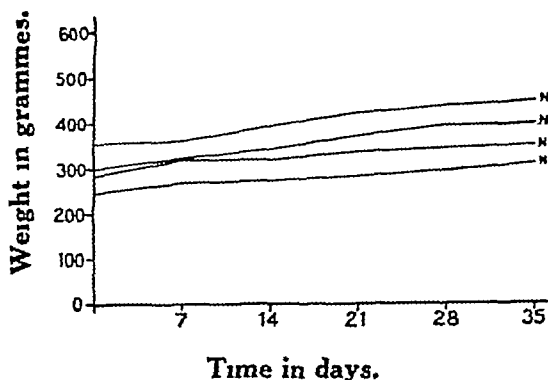
## GROUP I

*Negative controls*



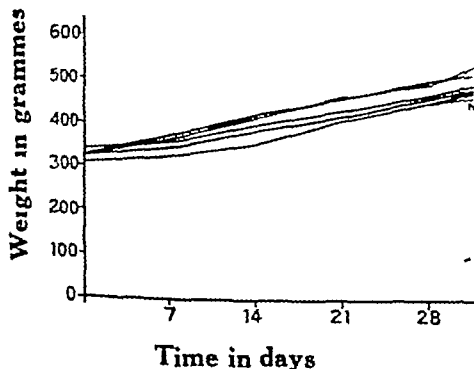
## GROUP II

*Basal diet plus vitamin C*



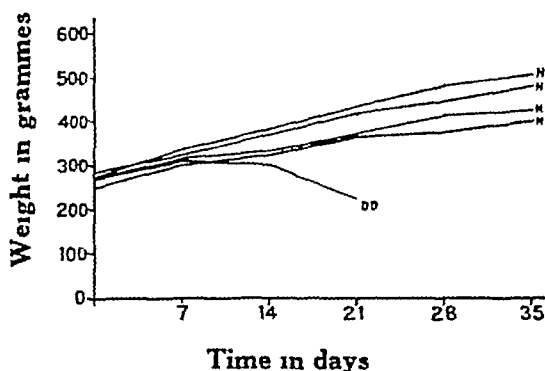
## GROUP III

*Basal diet plus Bengal gram*



## GROUP IV

*Basal diet plus Bengal gram*



N = normal

DS = died of scurvy

P = pregnant

DD = died showing diarrhoea

All the negative controls died within 21 to 28 days and post-mortem examination revealed typical signs of scurvy. All the positive controls as well as the animals in groups III and IV showed steady increase in weight, thereby confirming the chemical findings that the dry samples of Bengal gram contained

significant amounts of vitamin C. The amount of vitamin C received daily by the animals in groups III and IV varied from 1.5 mg. at the beginning of the experiment to 4.5 mg. at its close.

The guinea-pigs receiving Bengal gram as a source of vitamin C showed better growth than the animals in group II receiving 5 mg. of vitamin C per day in the form of pure ascorbic acid, which was more than that supplied by the diets containing the Bengal gram. The amounts of vitamin C ingested were adequate for maintenance and growth in both cases. The extra growth of the animals receiving Bengal gram was presumably due to the fact that the gram supplied other factors required by the guinea-pigs. Wooley (1942) recently showed that guinea-pigs require apart from vitamin C, three or more dietary factors besides those which are sufficient for the growth of species such as rats and mice. Euler and Klusmann (1932, 1933, 1933a) found that the titration value of germinated peas was lower than their anti-scorbutic value as tested biologically on guinea-pigs and this they attributed to the combination of ascorbic acid with 'Hemmungstoffe' which inhibits its reducing properties, but from which it is liberated by hydrolysis in the digestive tract of animals. Guha and Ghosh (1935) have made a similar observation in the case of germinated green gram. The better growth given by the diets containing the pulse is not due to the presence of dehydro ascorbic acid, which possesses anti-scorbutic activity. Bengal gram, green gram and cow-pea do not appear to contain dehydro ascorbic acid (Bhagvat and Rao, *loc cit*).

The more rapid increase in weight produced by the diets containing Bengal gram was felt to be a matter of interest and some further experiments to throw light on the problem were carried out. These gave further evidence that the growth-enhancing effect was not due to vitamin C.

Experimental groups were as follows —

- GROUP A 4 animals receiving the basal diet *plus* pure vitamin C
- GROUP B 4 animals receiving Bengal gram diet (50 per cent level)
- GROUP C 4 animals receiving a diet similar to diet II, the Bengal gram being treated so as to destroy vitamin C almost completely. Pure vitamin C was added to this diet.
- GROUP D 4 animals receiving the same as group C except that untreated cow-pea replaced Bengal gram.

In addition there were two negative controls receiving the basal diet only and two negative controls receiving respectively the same as groups C and D without the addition of pure vitamin C.

The sample of Bengal gram used contained 18.7 mg./100 g. of vitamin C in terms of the chemical test. The sample of cow-pea contained only a trace of the vitamin. In order to destroy vitamin C in Bengal gram, the powdered pulse was moistened with N/10 alkali and kept at room temperature for 2 hours. This brought about irreversible oxidation of about 90 per cent of the vitamin present. The alkali was then neutralized by dilute HCl. The amount of NaCl added to the diet was adjusted after making allowance for that formed by the interaction of acid and alkali.

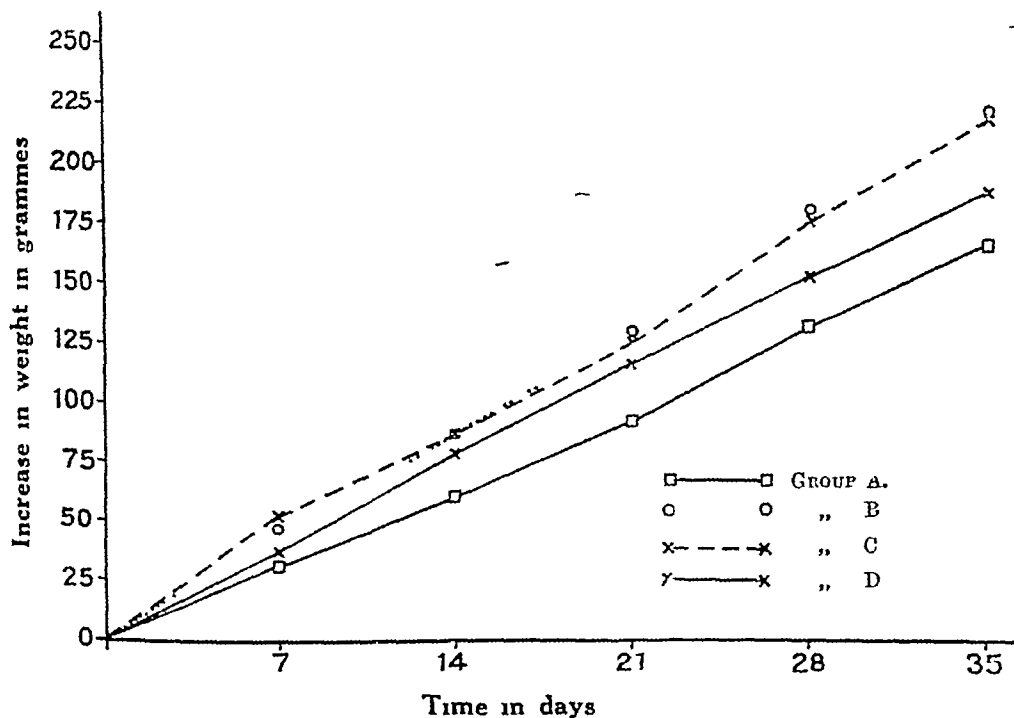
Weighed amounts of food were given daily and the quantity of vitamin C ingested daily by group II was calculated after weighing the food residue. The animals in groups A, C and D received this amount of vitamin C in the pure form. The protein content of the diets was as follows —

Groups	Per cent
A	19.3
B and C	17.3
D	20.0

The weight curves of the different groups are shown in Graph 2. The two animals receiving the basal diet only succumbed to scurvy in 3 to 4 weeks; their

GRAPH 2

*Weight curves of guinea-pigs receiving different diets*



weight curves are included in Graph 1. Those receiving diets C and D without vitamin C also died of scurvy, but the disease appeared more slowly and in less acute form, presumably owing to the fact that the diets contained traces of the vitamin.



The growth rate on the diets containing treated and untreated Bengal gram respectively was the same and more rapid than that produced by the basal diet plus vitamin C. This provides further evidence that the pulse contains a growth-promoting factor for guinea-pigs other than the anti-scorbutic vitamin. Similarly, the diet containing cow-pea produced better growth than the diet of group A, although the former contained approximately the same amount of vitamin C, the cow-pea supplying only additional traces of the vitamin.

The results suggest that pulses contain an alkali-stable growth-promoting factor for guinea-pigs. The nature of this factor is a question for further investigation. Bengal gram, cow-pea and other pulses, when treated with dilute alkali, give on warming an intense orange colour, which might be due to the presence of flavone or flavanone pigments. Rusznyak and Szent-Gyorgyi (1936) and Bentsath, Rusznyak and Szent-Gyorgyi (1936) claimed that citrin, which is a mixture of hesperidine and eriodectyol glucoside, and belongs to the flavanone group, and is found in lemon juice and red pepper, increased capillary resistance in guinea-pig receiving a scurvy-producing diet. The effect of citrin on the growth of guinea-pigs is not clear.

#### DISCUSSION

The biological experiments reported prove that samples of dry unsprouted Bengal gram contain significant amounts of vitamin C, sufficient to promote good growth in guinea-pigs when the pulse was included in the diet at 32 and 50 per cent levels respectively. Bengal gram appears to be somewhat exceptional among pulses in this respect, although green gram also gave a small titre in the unsprouted state. Fresh tender peas contain fairly large amounts of the vitamin but as they approach maturity their vitamin content diminishes until it approaches zero (Mack, Tressler and King, 1936). It would be of interest to investigate the vitamin C content of Bengal gram from the immature to mature stage, and to study the relation between the season of harvesting and the amount of vitamin formed.

It is known that the diet consumed by the masses in India is deficient in many respects, which is shown by the prevalence of various food deficiency diseases. Yet scurvy appears to be an uncommon deficiency disease, in spite of the fact that the diet of poor Indians is usually lacking in fresh fruits and vegetables. Severe scurvy has been observed in a famine area (Nicol, 1941, Khan, 1942) but otherwise cases or outbreaks of scurvy do not appear to have been reported within recent years. It may be that one of the reasons for the rarity of severe scurvy is the fact that dry Bengal gram contains some vitamin C, as reported in this paper. This pulse is consumed in various forms in most parts of the country throughout the year. Even when taken in relatively small quantities it might supply enough vitamin C to raise the vitamin C content of diets just above the danger point, i.e. it might help to satisfy *minimum* requirements for the prevention of outspoken clinical scurvy. Little information is available about the incidence of very mild 'sub-clinical' scurvy in India.

## SUMMARY

1 Dry samples of Bengal gram were found to contain significant amounts of vitamin C which were sufficient to promote good growth in guinea-pigs when fed at 32 and 50 per cent levels of intake respectively. This observation may have a bearing on the apparently low incidence of severe scurvy in India.

2 Evidence has been brought forward to show the presence in pulses of an alkali-stable growth-promoting factor for guinea-pigs.

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## VITAMIN C CONTENT OF TENDER WALNUT (*JUGLANS REGIA*)

BY

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[Received for publication, September 26, 1942]

THE Forestry Abstracts of 1941 (vol 2, p 236) contain an abstract of an article by Pechnikova (1940) in which reference was made to investigations carried out by a Russian worker, Gergelezhuu, on the vitamin C content of tender walnut. The abstract stated that 'the nuts of *Juglans regia* accumulate vitamin C during the first 20 days of their development until the vitamin C content of the kernel reaches 3.06 per cent' or 30.36 milligrams per gramme. This is a remarkably high figure, considerably in excess of any values so far reported in the literature for any fruit or vegetable. It was felt that it would be of value to confirm it, and to investigate the possibility of using tender walnuts as a source of vitamin C for practical purposes.

Ten pounds of immature walnuts were obtained through the courtesy of the Forest Department, Kashmir. These were of the size and shape of a small lemon with an average weight of 18 grammes. The fruit at this stage of maturity may be described as follows: there is the skin, which is thick and green in colour, beneath the skin there is the whitish pulp, and in the centre of the fruit a thin sac of irregular shape containing a brownish fluid. As the fruit matures, the pulp hardens into the characteristic woody shell and the fluid in the sac is gradually transformed into the nut.

Vitamin C was determined by the chemical method using the indophenol dye. Tests for interfering substances showed that these were present in amounts sufficient to raise the indophenol titre by 4 to 6 per cent. Values have been corrected accordingly.

The vitamin C content of whole immature fruits was found to be on the average 14.7 mg/g. The distribution of the vitamin in the component parts of a typical immature fruit was as follows —

*Distribution of vitamin C in the immature walnut*

	Weight, g	Weight expressed as percentage of fruit	ASCORBIC ACID		
			Total, mg	Percentage of the total amount present	Expressed as mg/g
Whole fruit	18.2		268.2		14.7
Skin	5.8	31.8	63.4	23.6	10.9
Pulp	8.8	48.4	204.8	76.4	23.3
Immature nut	3.6	19.8	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>

The pulp contains most of the vitamin C present in the whole fruit, while the immature nut is devoid of the vitamin.

These observations confirmed those of the Russian worker as regards the high vitamin C content of tender walnut, although the values obtained were lower than those reported by him. Experiments were undertaken to discover whether a dried or dehydrated powdered product, rich in anti-scorbutic activity, could be prepared from the immature fruit. The methods employed in dehydration and the vitamin C content of the dehydrated products were as follows —

Dehydration method	Time taken for dehydration, hours	Vitamin C content, mg/g
1 Sun drying	6	32.3
2 Oven drying at 150°F with air blast	2	57.9
3 Steam oven	3	48.9
4 Electric hot plate, moisture driven off by a fan	1	87.9
5 Tunnel drier (Marshall oven, 150°F)	11	38.9
6 Improvised tunnel and a blast of hot air from a universal hair drier	1½	49.4

The dehydrated material was very rich in vitamin C, the value obtained apparently depending to a considerable extent on the time taken for dehydration.

In the manufacture of dehydrated vegetables, the fresh material is usually 'blanched' for a short period either in boiling water or live steam under pressure. This improves the quality of the dehydrated product and steam-blanching may also have a preservative effect on vitamin C owing to destruction of oxidizing enzymes. When tender walnuts were blanched in boiling water for 3 minutes and subsequently dehydrated using method 6, the final product retained 0.4 per cent of the vitamin C initially present, while the water used for blanching contained 29.5 per cent. The percentage loss of vitamin C was thus 70.1. (In the case of vegetables, and in general, dipping in boiling water for a few minutes does not cause so considerable a loss.) When, however, the fresh material was blanched in live steam at about 15-lb pressure for 3 minutes, the loss of vitamin C was only 26.8 per cent and the dehydrated product obtained by method 6 was much richer in vitamin C. The highest value obtained was 106 mg/g, i.e. a concentration of over 10 per cent. This is a remarkably high figure, more than 3 times as high as that given by the richest preparations of dried amla (*Phyllanthus emblica* Linn.) prepared in these Laboratories.

Fruits in a more mature state were obtained for testing from a single tree which grows in Sims Park in Coonoor. It was found that most of the vitamin C present in the immature fruit disappears with the hardening of the pulp and the formation of the nut. The outer covering or skin, during the later stages of maturity, contained less than 1 milligram of vitamin C per gramme, while the shell and the nut were completely devoid of it. The disappearance of vitamin C appears to run parallel with the formation of fat in the nut, whether the former plays any part in the latter process is at present unknown.

Fairly mature walnuts, pickled or preserved in sugar solution, were sent for test by the Fruit Specialist, Punjab, Lyallpur. These were found to contain only traces of vitamin C.

In spite of the high vitamin C value of tender walnuts, their use as an anti-scorbutic in the fresh or dehydrated form does not appear to be very practicable. The tender fruit is very bitter, and the dried product is so repulsive in taste that it could scarcely be swallowed. Further, the vitamin is not stable on storage. Tender walnuts, stored in the refrigerator, lost 30 per cent of the vitamin in 3 weeks and the loss in the dehydrated material on storage at room temperature for a similar period was 68 per cent. It might be possible to devise methods of preparation (e.g. pickling) which would to some extent disguise the bitter taste, or alternatively, the vitamin might be extracted from the fruit by suitable means. But there are the practical disadvantages that the fruit would have to be obtained and processed at a certain brief stage of immaturity, the ripe fruit being devoid of the vitamin, and that fully mature walnuts are a valuable commercial crop.

#### SUMMARY.

1. Immature walnuts are a rich source of vitamin C, containing nearly 15 milligrams per gramme of fresh material. This is present mainly in the pulp. As the fruit ripens and the nut is formed, the vitamin disappears.

2 The use of tender walnuts as a source of vitamin C does not appear to be very practicable

#### ACKNOWLEDGMENT

Thanks are due to Mr J A Wilson, I F S, District Forest Officer, North Coimbatore, for drawing the attention of the Director, Nutrition Research Laboratories, Coonoor, to the abstract referred to

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## THE VITAMIN C CONTENT OF SOME DEHYDRATED VEGETABLES AND FRUITS, AND OF FRUIT JUICE PREPARATIONS

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[Received for publication, October 19, 1942]

EXPERIENCE in the British Navy in the eighteenth century showed that dried vegetables were without anti-scorbutic value. An interesting discussion of this subject will be found in the Medical Research Council Report—'Vitamins, a survey of present knowledge' (1932). Until recently, the only way of drying vegetables was by prolonged exposure to sun and air which would necessarily have a destructive effect on vitamin C. The same report states that sun-dried fruits may have a distinct, though feeble, anti-scorbutic activity.

The rapid dehydration of vegetables and fruits by modern methods is, however, essentially different from prolonged sun-drying. In this process the fresh material is 'blanched' or 'scalded' by steaming or dipping in boiling water for a few minutes and then exposed to a current of hot air in a suitable chamber and dehydrated in about 4 to 10 hours, the period depending on the nature of the material and other factors. Dehydrated vegetables can be 'reconstituted' by soaking in water or cooking and clearly the changes produced by mechanical dehydration are much less drastic than those produced by slow sun-drying. It was therefore felt to be of interest and value to study the effect of rapid dehydration on vitamin C content. In the present paper, the results of tests carried out on dehydrated vegetables and fruits are reported. The effect of storage on vitamin C content has also been investigated. In addition the results of tests in various samples of fruit juices are also given.

### METHOD OF ASSAY

The chemical method involving the use of 2,6-dichlorophenol-indophenol dye has been used throughout. Provided certain precautions are observed, this method is accurate and gives results which conform with those obtained by biological tests (Harris and Olliver, 1942).



## DEHYDRATED VEGETABLES

Samples of dehydrated vegetables were kindly supplied by Messrs Parry & Co, Ltd, Ranipet. The vitamin C content of a number of samples is shown in Table I. The fresh material before dehydration was not available for test and the values for the fresh vegetables included in the table are the results of tests with other samples.

TABLE I

*Vitamin C content of fresh and dehydrated vegetables*

Vegetables	Dehydrated, mg /g	Fresh, mg /g	Approximate percentage loss of vitamin C during de hydration
Cabbage ( <i>Brassica oleracea capitata</i> )	0.95	1.24	88
Cauliflower ( <i>Brassica oleracea botrytes</i> )	1.50	0.66	72
Carrot ( <i>Daucus carota</i> )	0.10	0.03	50
Turnip ( <i>Brassica rapa</i> )	0.08	0.43	93
Parsnip ( <i>Pastinaca sativa</i> )	0.10	0.16	79
Knol khol ( <i>Brassica oleracea caulorapa</i> )	1.40	0.85	83
Onion ( <i>Allium cepa</i> )	0.05	0.11	88
Brinjal ( <i>Solanum melongena</i> )	0.30	0.23	85
Bitter gourd ( <i>Momordica charantia</i> )	1.80	0.88	72
Ladies' fingers ( <i>Hibiscus esculentus</i> )	1.15	0.16	13
Potato ( <i>Solanum tuberosum</i> )	0.10	0.17	49

Losses occurring on dehydration cannot be exactly deduced from these figures, since the vitamin C content of the fresh vegetables used for dehydration was not known. If this corresponded with that of the fresh samples tested in

the Laboratories, losses, reckoned on a moisture-free basis, ranged from 50 to 90 per cent, except in the case of *Hibiscus esculentus*, where it was of a smaller order. This may have been due to the fact that the sample used for dehydration was exceptionally rich in vitamin C.

Many of the dehydrated vegetables were too low in vitamin C to be of practical value as a source of the vitamin in the quantities in which they are likely to be consumed. Five vegetables—cabbage, knol-khol, cauliflower, bitter gourd and ladies' fingers—gave higher values than the rest and were selected for further study. The effect of storage for 3 and 6 weeks at Coonoor room temperature (18°C to 23°C) and in an incubator at 37°C is shown in Table II. The material was stored in closed, but not sealed, cigarette tins.

TABLE II.

*Percentage loss of vitamin C in dehydrated vegetables on storage*

Dehydrated vegetables.	3 WEEKS' STORAGE		6 WEEKS' STORAGE	
	Room temperature	37°C	Room temperature	37°C
Cabbage	55	18	74	42
Knol khol	61		70	
Cauliflower	32	25	75	47
Bitter gourd	50	11	77	42
Ladies' fingers	74	44	76	48

The percentage loss after 6 weeks' storage was very considerable. Losses were actually greater in the material stored at room temperature than in the material stored in the incubator. The latter was dry and crisp whereas the former took up moisture, which may have accelerated destruction of the vitamin.

The stability of vitamin C in samples stored in sealed tins was then investigated. These were dehydrated after steam-blanching in the factory at Ranipet. Tins

containing dehydrated material\* manufactured simultaneously from the same original sample of fresh vegetable were stored at 37°C and one tin of each opened monthly for testing. The results are shown in Table III —

TABLE III

*Stability of vitamin C in dehydrated vegetables stored in sealed tins at 37°C*

Dehydrated vegetable	Initial vitamin C, mg /g	VITAMIN C CONTENT ON STORAGE, MG /G							
		1 month	Percentage loss	2 months	Percentage loss	3 months	Percentage loss	4 months	Percentage loss
Cabbage	0.70	0.48	32	0.40	43	0.35	50.0	0.27	60.7
Knol khol	0.40	0.30	25	0.23	44	0.20	50.0	0.20	50.0
Cauliflower	1.48	0.85	42	0.78	47	0.73	49.2	0.60	59.3
Bitter gourd	2.30	2.65	6	2.35	16	2.25	19.6	1.88	35.0

(After 3 to 4 months' storage in sealed tins, the samples of cabbage, knol khol and cauliflower developed an offensive smell and appeared unfit for use.)

Vitamin C was more stable in the material stored in sealed tins, but the loss was nevertheless fairly rapid. Some stability tests were also carried out on dehydrated potatoes. The quantity of dehydrated potatoes manufactured exceeds that of any other vegetable, and hence it is important that their nutritive value should receive special attention. Samples giving an initial value of 0.35 mg /g (a higher value than that recorded for the sample in Table I) were stored for 3 months at room temperature in closed tins. The loss of vitamin C during this period was only of the order of 10 per cent. It appears that vitamin C in dehydrated potatoes is somewhat more stable than in other dehydrated vegetables. Beckley and Notley (1911) reported a value of 0.49 mg /g for a sample of dehydrated potato after 7 months' storage.

*The effect of reconstitution and cooking*—The dehydrated vegetables reconstituted well on being soaked in water for a few hours, but they did not take up as

much water as they had lost on dehydration One gramme of dehydrated material 'reconstituted' to the following —

Cabbage	7 20 g	Bitter gourd	5 90 g
Knol-khol	9 76 g	Ladies' fingers	8 08 g
Cauliflower	5 85 g	Potato	3 60 g

These figures do not correspond with the amounts of the fresh vegetables necessary to yield 1 g of the dehydrated material calculated on the basis of their initial moisture content It appears that in the case of many vegetables the weight of the reconstituted vegetable may be a half or less of the original fresh weight During reconstitution some of the soluble material passes into the water Further, it is probable that dehydration leads to structural changes in the cells so that the material cannot be restored to its original consistency

In the cooking tests, the vegetables, with the exception of cabbage, were reconstituted and cooked by being placed in boiling water and cooked for one hour Dehydrated cabbage was soaked in minimal amounts of water for 2 hours, the water was then raised to the boil and kept boiling for 10 minutes The reconstituted vegetable and the water were both tested for vitamin C, the results being given in Table IV The effect of cooking the fresh vegetable is shown for purposes of comparison

TABLE IV

*Effect of cooking on the vitamin C content of dehydrated vegetables*

Vegetables	DEHYDRATED			FRESH		
	Vitamin C in cooked material, per cent	Vitamin C in cooking water, per cent	Loss during cooking, per cent	Vitamin C in cooked material, per cent	Vitamin C in cooking water, per cent	Loss during cooking, per cent
Cabbage	16	52	32	11	25	64
Knol khol	5	61	34	6	73	21
Cauliflower	3	40	57	9	81	10
Bitter gourd	19	9	72	28	11	61
Ladies' fingers	19	9	72	17	13	70
Potato	35	45	20	11	16	73

Results expressed as percentage of vitamin C originally present in uncooked dehydrated material.

In the case of cabbage, knol-khol, cauliflower and potato, most of the vitamin C passed into the cooking water. The total loss on cooking ranged from 20 to 72 per cent, or, to put it the other way round, the cooked material plus cooking water retained from 28 to 80 per cent of the vitamin originally present. It is clear that, if the cooking water is not consumed, the losses of vitamin C involved in the reconstitution and cooking of certain dehydrated vegetables are very considerable.

*The effect of 'blanching'* — 'Blanching' in this context means dipping the fresh vegetables in boiling water or subjecting them to live steam under pressure for 3 to 10 minutes. Blanching improves commercial quality and prevents staining, it may also have a preservative effect on vitamin C because the heat destroys the ascorbic-acid oxidase usually present in fresh vegetables (Beckley and Notley, *loc cit*). As regards vitamin C, steaming is likely to give better results, since vitamin C may be leached out when fresh vegetables are placed in boiling water. Seven samples of steam-blanching dehydrated vegetables, supplied by Messrs Parry & Co., were tested. In Table V the values obtained are compared with those given by other samples not subjected to steam-blanching (*cf* Table I). Further tests on samples from the same original batch of vegetables, steam-blanching and dehydrated in the Laboratory, by an improvised apparatus (Sekhon, 1942), gave results of the same order.

TABLE V.

*Effect of steam-blanching on vitamin C content.*

Vegetables	Steam blanched Vitamin C, mg /g	Without steam blanching Vitamin C, mg /g
Cauliflower	2 35	1 50
Brinjal	0 18	0 30
Carrot	0 25	0 10
Parsnip	0 15	0 10
Ladies' fingers	1 20	1 15
Potato	0 35	0 10
Bitter gourd	2 90	1 80

In the case of potato, carrot, bitter gourd and cauliflower the steam-blanching samples gave definitely higher values. In a series of experiments carried out in the Laboratories, dehydrated potatoes steam-blanching at about 15-lb pressure gave consistently higher values than potatoes blanched in boiling water or not subjected to the blanching process. The steam-blanching of whole unpeeled potatoes for 10 minutes gave better results than the steam-blanching of such potatoes for shorter periods. With peeled and sliced potatoes, 3 minutes' steam-blanching was found to be suitable. Employing steam-blanching along these lines and rapid dehydration, it was found possible to obtain dehydrated potatoes containing 0.75 mg/g of vitamin C. With more prolonged drying, values were considerably lower. Even under the best laboratory conditions there was a loss of about 25 per cent of vitamin C during dehydration.

*Effect of 'scalding'*—It is generally recognized that the preliminary 'scalding' of vegetables by dipping them in boiling water for a few minutes before dehydration minimizes subsequent losses of vitamin C. This was confirmed by tests on cabbage, potato and carrot in the Laboratory, in which the materials were subjected to preliminary dipping for 3 minutes either in boiling water alone or in boiling 0.8 per cent sodium-sulphite solution, equivalent to 0.2 per cent  $\text{SO}_2$ . The values given by 'scalded' samples after dehydration were 10 to 25 per cent higher than those given by samples not subjected to this preliminary treatment.

Certain commercial samples of potato dehydrated after being dipped in boiling water and subsequently in 1.5 per cent sodium-sulphite solution were found to be completely devoid of vitamin C. It is not clear why this result was obtained, but it is important to note its occurrence.

*Effect of sulphur fumes*—Fumigation with sulphur fumes is sometimes applied to the fresh material before dehydration to prevent staining. A series of tests showed that the treatment did not affect vitamin C values.

*The practical value of dehydrated vegetables as anti-scorbutics*—Modifications in manufacturing technique might make it possible to produce dehydrated vegetables giving vitamin C values of a somewhat higher order than those reported above. For example, it is probable that the more rapid the process of dehydration, the less the destruction of vitamin C which is likely to take place, provided the temperature does not exceed certain limits. It appears that certain dehydrated vegetables may be eliminated as anti-scorbutics, but there are others which have a definite though limited, value. The losses which take place on cooking or storage are of special importance. Assuming that the cooking water is consumed, 2 oz (dehydrated weight) of the following vegetables, after 2 months' storage in sealed tins at  $37^\circ\text{C}$ , would supply approximately the amount of vitamin C indicated—

Vegetables	Vitamin C (mg)	Vegetables	Vitamin C (mg)
Cabbage	14 to 17	Cauliflower	18 to 25
Knol-khol	10 to 20	Bitter gourd	15 to 30
Potato	12 to 15		

The value for potato is based on the assumption that the initial vitamin C content after dehydration was 0.35 mg/g. It is to be observed, however, that some commercial samples of dehydrated potatoes may be entirely devoid of vitamin C.

Two oz. are roughly equivalent to 8 oz. to 12 oz. of the reconstituted material. The amounts of vitamin C which could be obtained from an intake of this order are by no means negligible and might serve to turn a scurvy-producing diet into a diet which protected against the disease. Nevertheless, it is clear that dehydrated vegetables prepared by ordinary commercial methods cannot be regarded as safe or potent anti-scorbutics. Methods of manufacture are not completely standardized and while a dehydrated vegetable produced by one manufacturer might contain some vitamin C the same material processed in a different manner by another manufacturer might give a lower value or be devoid of the vitamin. Storage in air-tight containers and use as soon as possible after manufacture are to be recommended. The destruction of vitamin C on cooking can probably be reduced by cooking for minimum periods and by the addition of an acid substance, such as tamarind, to the cooking water.

#### DEHYDRATED FRUITS

*Amla* (*Phyllanthus emblica* Linn.) — *Amla* is available in unlimited quantities in India as a minor forest product. It is very rich in vitamin C, coming second only to tender walnut (Pechnikova, 1940, quoted by Ranganathan, 1942) in this respect among fruits hitherto tested. The fresh pulp of the ripe fruit contains 4 mg. to 8 mg. of vitamin C per gramme, most of which is present in the juice, an average sample of the latter containing 5 mg./ml. to 7 mg./ml. Unripe fruit, in which the kernel is scarcely formed, is relatively poor in the vitamin (0.5 mg./g. to 0.7 mg./g.).

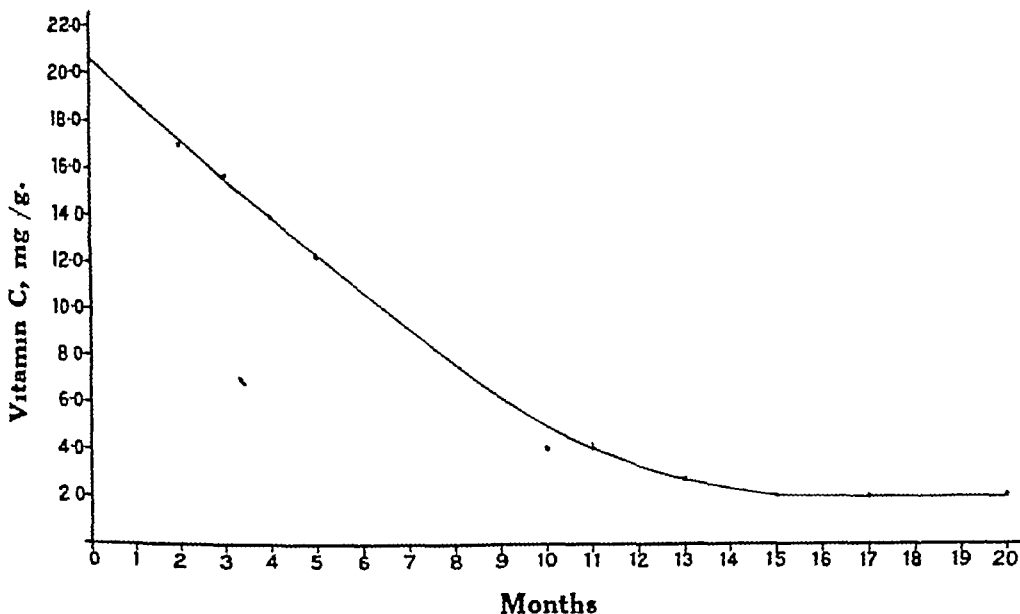
*Amla* pulp can be dehydrated and powdered and remain rich in vitamin C. One of the reasons for the relative stability of the vitamin in this fruit is that it contains tannins which partially protect the vitamin from destruction. The dehydration of *amlam* pulp reduces its bulk by about 8 times. If no vitamin C were destroyed in the process, the average vitamin C content of the powder would be of the order of 50 mg./g. Actually this theoretical value is never attained. Sun-dried *amlam* pulp usually contains 10 mg./g. to 14 mg./g., the highest value being about 19 mg./g. If drying in the open air is prolonged for more than 4 to 8 hours, the values obtained fall rapidly.

Mechanical dehydration produces better results. In this process the pulp is broken up into small fragments and dried on trays in a blast of hot air. Usual values for the dehydrated material are 24 mg./g. to 28 mg./g., the highest recorded in the Laboratory being 35 mg./g. Mechanical drying at about 93°C. for 20 to 30 minutes produced the best results. As far as possible the fruit should be kept out of contact with metal during the process of pulping and dehydration.

*Stability* — Vitamin C in dehydrated *amlam* powder is stable for 3 months at about 2°C., but storage at this temperature for 6 months involves a loss of about

10 per cent Losses in 3 months at Coonoor room temperature ( $18^{\circ}\text{C}$  to  $23^{\circ}\text{C}$ ) and at  $37^{\circ}\text{C}$  respectively were of the order of 20 to 25 per cent Stability is somewhat increased when the powder is punched into tablets The loss of vitamin C in amla powder stored at  $37^{\circ}\text{C}$  (not *in vacuo*) for a period of 20 months, as determined by tests at regular intervals, is shown in the Graph —

GRAPH



Loss of vitamin C in a sample of dried amla powder stored at  $37^{\circ}\text{C}$ , not *in vacuo*

The destruction of vitamin C is reduced by storage *in vacuo*, the loss in these circumstances at  $37^{\circ}\text{C}$  in 6 months being only 10 to 15 per cent Even *in vacuo*, however, loss proceeds steadily on prolonged storage

Amla powder stored in a cold room for 8 months was successfully used by Khan (1942) in the treatment of severe cases of human scurvy

*Note on the estimation of vitamin C in the presence of tannins*—According to Mirmanoff and Toledo (1940), the presence of tannins interferes with vitamin C tests carried out by the chemical method with 2,6-dichlorophenol-indophenol Since amla is rich in tannins, attention was given to this point Ample evidence was obtained that any interference by tannins in the reaction is eliminated when the test is carried out in a strongly acid medium, at or below pH 3, and when the end point is taken as the stage at which the pink colour remains unchanged for about 15 seconds This is in general agreement with the findings of Bessey (1938) Damodaran and Nair (1936) observed that there are 2 tannins present in amla, one of these does not react with the dye while the other gives a slight reaction,



1,000 mg of pure tannin giving the effect of 2 mg to 3 mg of ascorbic acid. The vitamin C values given by amla were found to be consistently dependent on such factors as the method of drying, period of storage, etc., which do not affect tannin content. Samples of amla powder quite devoid of vitamin C but containing the usual amount of tannins have been tested from time to time. Further, the results of chemical tests on amla were checked by biological assays on guinea-pigs.

Tannins are quantitatively removed by mercuric acetate. Treatment with mercuric acetate, following the technique of Emmerie and van Eekelen (1934), reduced the titration value given by amla powder by 2 to 10 per cent, with an average of 5 per cent. It is unlikely that this slight difference was due to removal of tannins.

It may be added that wattle-bark, which is rich in tannins, does not react with the dye in acid medium below pH 3. A mixture of equal parts of amla powder and wattle-bark gave the same titre as the same quantity of amla powder alone. The fresh green leaves of the tea shrub give a fairly high vitamin C titre by the chemical method. Processed tea leaves, containing bulk for bulk much larger quantities of tannin, give no titre.

*Country guava* (*Psidium guajava*) —The country guava is a rich source of vitamin C, the value of 2.99 mg/g for the fresh pulp having been reported by the author (Ranganathan, 1935). Levy and Fox (1935) found lower values (0.56 mg/g to 0.89 mg/g) but Goldberg and Levy (1941) have recently reported a figure of 2 mg/g to 3 mg/g, which corresponds to that reported by the author. Goldberg and Levy (*loc cit*) claim to have manufactured, from country guava, a dried powder containing about 30 mg/g of vitamin C. By their method the fresh fruit was blanched for 2 minutes and then dried at 130°F (55°C). Numerous samples of dried guava were prepared both in the Laboratory and in the factory, in general following the method described by the above workers. The vitamin C value of the majority of the dried preparations ranged from 2 mg/g to 5 mg/g. Eventually, by rapid dehydration in the Laboratory after preliminary dipping in boiling sodium-sulphite solution (equivalent to 0.2 per cent SO<sub>2</sub>) for 3 minutes, a sample was obtained giving a value of 22.8 mg/g. The powder was attractive in appearance and palatable. Unfortunately, vitamin C potency rapidly fell on storage, some 30 per cent of the vitamin being lost on storage for 24 hours both at room temperature and in the incubator. The vitamin would probably be more stable in guava powder or tablets packed *in vacuo*, but this is not feasible at the present time owing to the shortage of tin. Guava powder with a potency of 20 mg/g to 30 mg/g would, if sufficient stability could be assured, make an admirable anti-scorbutic and it is desirable that further work on the subject should be carried out. At the moment, however, guava powder cannot be recommended as a practicable anti-scorbutic.

*Cashew apple* (*Anacardium occidentale*) —Cashew apple is rich in vitamin C and available in fair abundance in India. The whole fruit contains about 3.2 mg/g and its juice, in which the vitamin is principally present, about 3.5 mg/ml. Slices of the fruit dried in the sun for 7 to 10 hours gave values ranging from 10.5 mg/g.

to 16.25 mg/g. A crisp easily-powdered material could not, however, be produced even with prolonged sun-drying. Material dried in an oven for 4 hours at 70°C was devoid of vitamin C.

Sun-dried cashew apple lost nearly 50 per cent vitamin C in a week whether stored at room temperature or at lower temperatures. The dried slices did not reconstitute well on soaking in water and most of the vitamin C passed into the water in which reconstitution took place. The dried fruit has a strong acid taste. Since the season for cashew apple is short and supplies are localized, collection of the fruit, processing, powdering of the dehydrated material, etc., would present a formidable problem. Even if these difficulties were surmounted, the poor stability of vitamin C in the dried cashew apple rules it out as a practicable anti-scorbutic.

*Papaya and pineapple*—Papaya (*Carica papaya*) and pineapple (*Ananas sativus*) are two common fruits of India which contain fair amounts of vitamin C. Slices of these fruits when oven-dried at 70°C yielded a product which was fairly palatable but completely devoid of vitamin C. Sun-dried material contained only traces of the vitamin. Commercially dehydrated pineapple contained 0.35 mg/g of vitamin C.

*Fruit juices and fruit juice concentrates*—Fruit juice preparations have often in the past been used as anti-scorbutics. The fresh juice of such fruits as lime, orange and pineapple is rich in vitamin C and when preserved and bottled such juices are very agreeable to take. Unfortunately vitamin C is very unstable in fruit juices. For example, lime juice was found to lose all its vitamin C when stored at 37°C for one month. In cold storage at 2°C the loss during the same period was 50 per cent and in 45 days 88 per cent. The vitamin was found to be somewhat more stable in orange juice, but rapid loss was observed in this case also. Preservatives, such as salicylic acid, benzoic acid, boric acid, absolute alcohol and sodium metabisulphite, had little stabilizing effect. Bottled fruit juices available on the market were found to contain little or no vitamin C.

Fruit juice concentrates can be prepared by distillation under reduced pressure. Fairly potent concentrates were prepared in the Laboratory, but it was found that the destruction of vitamin C during the process was considerable, amounting to 75, 71 and 85 per cent respectively with lime, pineapple and cashew fruit juice. No doubt losses could be greatly minimized by carrying out the concentration in an atmosphere of an inert gas, e.g. nitrogen, but this would probably not be feasible on a commercial scale in India at the present time. Various fruit juice concentrates prepared in India were sent to the Laboratories for test. These in general were found to have a vitamin C content only a little higher than the average value of the fresh juice from which they were prepared. The vitamin C present was unstable, from 40 to 60 per cent disappearing on storage for 3 weeks. Most of the samples readily fermented on storage.

In general there is little to be said in favour of bottled fruit juices and concentrates as anti-scorbutics. In relation to their vitamin content juices are bulky and

hence unsuitable for transport and the vitamin in both juices and concentrates is highly unstable

#### SUMMARY

1 An investigation of the effect of dehydration on the vitamin C content of various vegetables and fruits is reported

2 The dehydration of vegetables in general destroys most of the vitamin C present in the fresh material. Certain vegetables however, retain vitamin C in appreciable quantities after dehydration

3 The vitamin C content of dehydrated vegetables falls rapidly on storage

4 Reconstitution and cooking lead to further losses of the vitamin remaining in dehydrated vegetables. Much of the vitamin C that survives passes into the cooking water

5 Amla fruit after dehydration remains rich in vitamin C, which is *relatively* stable in amla powder and tablets. Steady loss, however, occurs on storage. The presence of tannins in amla does not interfere with the estimation of vitamin C

6 It was not found possible to prepare a stable powder rich in vitamin C from country guava. Similar experiments with cashew apple, papaya and pineapple were also unsuccessful

7 Fruit juice and fruit juice concentrates cannot be recommended as anti-scorbutics, the vitamin in such preparations being very unstable

#### ACKNOWLEDGMENT.

The co-operation of Mr S G Davis and other members of the staff of Messrs Parry & Co in these investigations is gratefully acknowledged

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## THE EFFECT OF DEHYDRATION AND RECONSTITUTION ON THE CAROTENE CONTENT OF CERTAIN VEGETABLES

BY

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[Received for publication, October 19, 1942]

THE dehydration of vegetables leads to considerable destruction of the vitamin C present in the fresh material (Ranganathan, 1942). Fresh vegetables contain, in addition to vitamin C, another important vitamin, namely, pro-vitamin A or carotene, and the greater part of the vitamin A activity of most human diets is usually derived from the carotene present in vegetables. It is, therefore, important that the effect of dehydration should be studied in the case of this vitamin also. No work on this subject appears to have been reported in the literature.

Fresh vegetables rapidly lose carotene on storage (De, 1936), a high percentage of the carotene originally present being destroyed within a few days. The loss was found to be most marked in green leafy vegetables, which are in general rich in carotene. Numerous workers have shown that the sun-drying of grass and other fodder crops leads to destruction of carotene, which undergoes decomposition when exposed to ultra-violet and visible light (Dann, 1933, Guilbert, 1935). When drying is prolonged, enzymic and bacterial action also produces a destructive effect. On the other hand, carotene is more stable in fodder crops which are rapidly dried by mechanical means, e.g. in a hot chamber under a blast of hot air. Russell (1929), who carried out experiments on the effects of various methods of drying on the carotene content of alfalfa, reported that machine-dried material had a vitamin A potency at least 7 times greater than that of a field-dried sample. He did not, however, estimate the loss occurring during machine-drying.

In this paper the effect of dehydration and reconstitution on the carotene content of 13 vegetables is reported. By dehydration is meant rapid drying in a current of hot air, the dehydrated material being in such a state that soaking in water restores it almost to its original shape, appearance and consistency. The results of some preliminary tests on the effect of storage are also recorded.

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## METHOD OF DEHYDRATION

Commercially vegetables are dehydrated by being cut into thin slices and placed on trays in a large tunnel through which hot air is blown by fans. The process usually takes 6 to 12 hours, depending on the nature of the vegetable and the efficiency of the plant. In the Laboratory it was imitated by using an electric hair-drier, which delivered a current of hot air at about 70°C through a thick cardboard tube. The inedible portions of the vegetables were removed and slices of suitable size cut and placed in the tube. This makeshift apparatus proved an excellent dehydrator. The slices were occasionally stirred so that fresh surfaces could be presented to the air current. Dehydration was judged to be complete when the slices were crisp and broke cleanly on slight finger pressure. At this stage the moisture content was between 5 and 10 per cent. The time required for dehydration was from 3 to 5 hours.

Potatoes, when sliced and dehydrated without preliminary treatment, usually develop brownish stains, presumably as a result of enzyme action. Preliminary 'blanching' by steaming, or by dipping the potatoes in boiling water, prevents this undesirable change and such treatment is invariably applied in manufacture. In the present experiments, the potato slices were blanched in steam at about 15-lb pressure for 10 minutes previous to dehydration. This treatment was found to prevent change in colour. Most samples of other vegetables were dehydrated without preliminary treatment, but a number of samples were 'scalded' by being dipped in boiling sodium-sulphite solution before dehydration to observe the effect of this treatment.

## CAROTENE ASSAY

The method employed was the 'phase-separation' test described by Palmer (1922) and subsequently employed by De (1936a). One g to 10 g and 0.5 g to 2.0 g of fresh and dehydrated vegetables respectively were crushed with powdered glass in a porcelain mortar and repeatedly extracted with small quantities of absolute alcohol, until no more colouring matter could be removed. The alcohol extract was brought to about 88 per cent strength and shaken with small amounts of petroleum ether (B.P. 40°C to 80°C) until the petroleum ether layer became colourless. The petroleum ether extracts were pooled and washed consecutively with 90, 80 and 70 per cent alcohol to remove xanthophylls, rhodoxanthin, etc. The extract was washed well with water to remove all traces of alcohol. It was then dried over anhydrous sodium sulphate and shaken with dry precipitated  $\text{CaCO}_3$ . This operation removed chlorophyll and other non-hydrocarbon pigments.

Preliminary experiments on 3 vegetables showed that saponification at any stage is unnecessary. This is because vegetables do not contain appreciable quantities of fat and because the xanthophyll esters are either present in negligible amounts or else are removed by treatment with  $\text{CaCO}_3$ . The yellow solution containing only carotene was filtered and the filtrate made up to a definite volume. The intensity of the yellow colour was determined using a Lovibond tintometer. The amount of carotene present was then estimated by reference to a standard.

curve previously drawn showing the relation between yellow units and the strength of a solution of pure  $\beta$ -carotene

Carotene is present in vegetables mainly as  $\beta$ -carotene (Mackinney, 1935) No attempt was made to separate  $\alpha$ -,  $\beta$ - and  $\gamma$ -carotenes, the total yellow colour being compared with the standard The results have been stated as micrograms of carotene per 100 g of moisture-free material For translation into International units of vitamin A, one microgram of carotene may be taken as approximately equivalent to one International unit (De, 1936a)

It is to be observed that in carrying out carotene tests on small samples of vegetables, some sampling error is inevitable Every effort was, however, made to choose representative samples

#### THE EFFECT OF DEHYDRATION

The carotene value of the fresh sample was estimated and its moisture content determined The same sample was then dehydrated and tested in the same way The values were compared on a moisture-free basis Usually, the dehydrated samples were tested on the day following the test on the fresh samples, the former being stored in tins in a desiccator covered with a black cloth to protect them from light and atmospheric moisture The results are shown in Table I —

TABLE I

*The loss of carotene resulting from the dehydration of vegetables*

Vegetable	Botanical name	Micrograms of carotene per 100 g of fresh (moisture free) vegetable	Micrograms of carotene per 100 g of dehydrated (moisture free) vegetable	Percentage loss of carotene on dehydration
Cabbage (outer green leaves)	( <i>Brassica oleracea capitata</i> )	26,960	24,140	10.4
Fenugreek leaves	( <i>Trigonella foenum-graecum</i> )	36,990	32,490	12.1
Paralei	( <i>Petroselinum sativum</i> )	53,380	51,570	3.4
Spinach	( <i>Spinacia oleracea</i> )	34,290	32,280	2.9
Carrot	( <i>Daucus carota</i> )	50,350	49,220	2.2
Potato	( <i>Solanum tuberosum</i> )	162	85*	47.7
Bitter gourd	( <i>Momordica charantia</i> )	2,667	2,495	6.4
Cauliflower	( <i>Brassica oleracea botrytes</i> )	405	397	1.9

\* Steam blanched for 10 minutes before dehydration

TABLE I—concl'd

Vegetable	Botanical name	Micrograms of carotene per 100 g of fresh (moisture free) vegetable	Micrograms of carotene per 100 g of dehydrated (moisture free) vegetable	Percentage loss of carotene on dehydration
Cluster beans	( <i>Cyamopsis psoraleoides</i> )	2,894	2,422	16.3
French beans	( <i>Phaseolus vulgaris</i> )	3,182	2,724	14.1
Ladies' fingers	( <i>Hibiscus esculentus</i> )	518	450	13.1
Peas, English	( <i>Pisum sativum</i> )	1,752	1,536	12.3
Yellow pumpkin	( <i>Cucurbita maxima</i> )	14,240	14,180	0.4

The percentage loss of carotene was small in the case of carrot, yellow pumpkin, bitter-gourd, cauliflower and spinach. In cabbage, French beans, cluster beans, peas, parsley, fenugreek and okra it ranged from 10 to 20 per cent. Potato showed the greatest loss, which was presumably due to the preliminary steaming. Guilbert (*loc cit*) observed that the autoclaving of fresh vegetables caused considerable loss of carotene. The carotene content of fresh potatoes is low and the loss occurring on steaming and dehydration cannot be regarded as being of much practical importance.

In the factory a usual practice is to 'scald' vegetables before dehydration by preliminary dipping for a few minutes in boiling sodium-sulphite solution (equivalent to 0.2 per cent  $\text{SO}_2$ ). The effect of this treatment was investigated in the case of samples of cabbage, parsley and carrot. The retention of carotene in these vegetables on dehydration after preliminary 'scalding' was of the same order as that observed in dehydrated samples not subjected to 'scalding'. The effect of various methods of 'blanching' and 'scalding' on the carotene present in different kinds of vegetables requires further investigation.

#### THE EFFECT OF RECONSTITUTION

Dehydrated vegetables must be reconstituted before they are consumed. There are two methods of reconstitution which may be followed. The vegetable may be soaked in cold water for about 12 hours, during which it absorbs water and resumes its former appearance in the manner which is so surprising when seen for the first time, and then boiled for a short period. Alternatively, the vegetable is simply boiled for 20 to 50 minutes, the time depending on the nature of the vegetable. Boiling in this way produces satisfactory reconstitution, and because of its convenience is the method likely to be followed in practice. In the present experiments the second method of reconstitution was followed. The dehydrated

vegetables were boiled in sufficient quantities of water in beakers and the reconstituted vegetable removed from the cooking water. Both the water and the vegetable were then tested for carotene. The results are given in Table II. The samples of cabbage, carrot, bitter gourd and yellow pumpkin used in this investigation were manufactured by Messrs Parry & Co., Ltd., Ranipet, and sent to Coonoor for vitamin C testing. When the carotene tests recorded in Table II were carried out they had been in storage in the Laboratory for some weeks in closed unsealed tins at room temperature. The sample of cabbage used in this case had a much lower carotene value than that of the sample tested for losses on dehydration (Table I). The latter consisted of green outer cabbage leaves, whereas the former included the white inner part of the cabbage which is relatively poor in carotene.

TABLE II

*Loss of carotene occurring during the reconstitution of dehydrated vegetables*

Vegetable	Micrograms of carotene per 100 g of dehydrated vegetable	Micrograms of carotene per 100 g of dehydrated vegetable on reconstitution	Percentage loss of carotene on reconstitution.
Cabbage	750	672	10.4
Fenugreek leaves	29,010	28,890	0.4
Spinach	31,427	28,180	10.3
Carrot	82,640	80,870	2.1
Potato	78	70	10.2
Bitter gourd	5,981	4,838	19.5
Cauliflower	355	375	Nil
Cluster beans	2,230	2,000	10.3
Peas, English	1,375	1,180	19.8
Yellow pumpkin	7,750	7,250	6.5



The losses on reconstitution were not very serious. No carotene could be detected in the water used for boiling.

#### THE EFFECT OF STORAGE

The results reported above show that in general most of the carotene in vegetables survives dehydration and reconstitution. It is, however, necessary to know whether carotene is reasonably stable in the dehydrated material. In practice dehydrated vegetables will usually be consumed several months after manufacture.

Systematic tests of the effect of storage on carotene values would have to be extended over a period of 6 months or more. Some preliminary tests have been carried out on samples manufactured by Messrs Parry & Co., and on samples prepared in the laboratory, stored in unsealed tins at room temperature in Coonoor (18°C to 23°C). The initial value on manufacture of the former samples was unknown. The results, which are shown in Table III, in general indicate that while some loss does take place on storage, carotene in dehydrated vegetables is reasonably stable. For example, dehydrated carrots after storage for 3 months contained nearly 50,000 micrograms of carotene per 100 g. On the other hand, the carotene present in the sample of cauliflower tested was considerably reduced on storage for 3 months.

TABLE III.

*Carotene in dehydrated vegetables stored for various periods*

Vegetable	Period of storage, days	Carotene, $\mu\text{g}/100\text{ g}$
Parsley	0	51,570
	44	48,270
Carrot	34	64,000
	97	48,110
Potato	35	50
	98	50
Bitter gourd	37	3,450
	97	2,290

TABLE III—*concl'd*

Vegetable	Period of storage, days	Carotene, $\mu\text{g}$ /100 g
Cauliflower	36	400
	97	166
Cluster beans	0	2,422
	35	1,803
	72	1,722
Ladies' fingers	0	450
	30	384
	66	346
Peas, English	0	1,536
	34	1,464
	72	1,369
Yellow pumpkin	50	4,900
	80	4,358

## DISCUSSION

It is of interest to note the high carotene values given by dehydrated green cabbage, carrot, spinach and yellow pumpkin. Weight for weight, such vegetables when dehydrated may retain from one third to one half the vitamin A activity of an average sample of cod-liver oil. While before consumption their bulk is greatly increased by reconstitution, for purposes of storage and transport they may be regarded as concentrated sources of vitamin A.

## SUMMARY

1 The effect of dehydration and reconstitution on the carotene content of various vegetables has been studied.

2 Losses on dehydration are not serious, ranging from almost *nil* to about 15 per cent. The loss in a sample of potato subjected to steam-blanching before

dehydration was considerably greater. Reconstitution by cooking leads to losses ranging from almost *nil* to 20 per cent.

3 The carotene content of dehydrated vegetables appears to be reasonably stable on storage.

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## THE APPLICATION OF THE CYANOGEN BROMIDE TEST TO A STUDY OF THE METABOLISM OF NICOTINIC ACID IN RABBITS

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[Received for publication, September 26, 1942]

SINCE the identification of nicotinic acid with the pellagra-preventive vitamin (Elvehjem, Madden, Strong and Wooley, 1937, Spies, Cooper and Blankenhorn, 1938), various studies have been made of the metabolism of nicotinic acid in human beings and experimental animals (Swaminathan 1939, 1941, Shourie and Swaminathan, 1940, Harris and Raymond, 1939, Winegar, Pearson and Schmidt, 1940, Dann and Kohn, 1940, Dann, 1941). These have indicated that rats and sheep do not require an extraneous source of nicotinic acid and are presumably able to synthesize it. Dogs, guinea-pigs and monkeys, on the other hand, require nicotinic acid in the diet. The only animal which has hitherto been used for biological tests is the dog.

In the present investigation a study of nicotinic acid metabolism in the rabbit has been undertaken. This was felt to be of interest since (a) it would throw light on the nicotinic acid requirements of this species and (b) might suggest a technique for the biological assay of nicotinic acid using the rabbit as the experimental animal. In view of the criticism by Dann (*loc cit*) of the method used for the estimation of nicotinic acid in the experiments on the metabolism of nicotinic acid referred to above (Shourie and Swaminathan, *loc cit*), a re-investigation of the cyanogen bromide method as applied to urine has been undertaken.

### EXPERIMENTAL

The work falls roughly under three heads: first, a re-investigation of the cyanogen bromide method as applied to urine, second, an investigation of the available methods for the estimation of trigonelline in urine, and third, a study of the metabolism of nicotinic acid in rabbits.

### SECTION A

#### *The cyanogen bromide method applied to urine*

Since the publication of the author's method for urine (Swaminathan, 1939) various modifications have been described by different workers (Harris and

Raymond, *loc cit*, Melnick and Field, 1940, Bandier, 1939, Perlzweig, Levy and Sarett, 1940) These include the use of different reagents for the hydrolysis of nicotinic acid derivatives into nicotinic acid and also for the decolorization of the extracts, and the use of different aromatic amines in the colorimetric procedure. The method used by the author in the rat experiments (Shourie and Swaminathan, *loc cit*) has been criticized by Dann (*loc cit*) as follows 'Charcoal was used in treating urine hydrolysates and extracts of solid matter and the extracts were not fully decolorized before the Komig method was applied, each of these points means the introduction of serious error' These points have been repeatedly investigated in this Laboratory and the reliability of the technique evolved compared with that of the methods suggested by other workers. It has been shown that charcoal in an alkaline medium does not adsorb nicotinic acid from urine solutions, and that the presence of residual colouring matter does not interfere with the accuracy of the test when it is allowed for by 'blank' estimations under the conditions of the test as described. The results of further experiments to substantiate these points are given, below —

The method described previously by the author has been compared with that of Perlzweig *et al* (*loc cit*). The results obtained by the two methods correspond well. In trying to apply the methods to rabbit urine, it was found that the preliminary removal of interfering substances by treatment of the urine with basic lead acetate was necessary, as otherwise the final extracts were deeply coloured.

*Treatment of urine with lead acetate* — The urine and washings, equivalent to the weekly collection from 3 rabbits, were adjusted to pH 7 and treated with a slight excess of basic lead acetate to precipitate colouring matter, protein derivatives, etc., and the excess of lead was removed as the sulphate. The extract, after being adjusted to pH 10 (using phenolphthalein as internal indicator), was evaporated to a small bulk (about 150 ml). It was cooled, brought to pH 10 and centrifuged. The clear centrifugate was carefully separated, the residue was washed once with 100 ml of N/10 NaOH and the washings added to the main bulk. The volume was made up to 420 ml, so that 20 ml of this extract represented the daily average urinary output of one rabbit. The procedures of Swaminathan (1939) and Perlzweig *et al* (*loc cit*) were then applied as described —

*Procedure I* (Method described by Swaminathan slightly modified)

The author originally used 2 N NaOH for the hydrolysis of the nicotinic acid derivatives in urine and the duration of hydrolysis varied from 1½ to 3 hours. Later investigations have shown that hydrolysis with N NaOH for a period of 30 minutes was sufficient for complete hydrolysis. The modified method was as follows —

Four ml of 10 N NaOH and 16 ml of distilled water were added to 20-ml quantities of the extract prepared as described above. The mixture was boiled for about 10 minutes to decompose the urea and ammonium salts present and then heated in a boiling water-bath for 20 minutes. It was then cooled, first adjusted to pH 7 by the addition of glacial acetic acid and then made slightly alkaline (N/20 NaOH) by the addition of 0.4 ml of 10 N NaOH. Norit charcoal (0.5 g) was then added, the mixture heated in a water-bath with stirring for 10 minutes and filtered

hot The filter-paper and charcoal were washed three times with hot water (30 cc in all) The filtrate was adjusted to pH 7 and made up to volume (100 ml to 200 ml, depending on the concentration of the nicotinic acid present) Ten-ml aliquots were used for the colorimetric estimation of the nicotinic acid under the conditions described before (Swaminathan, 1939)

*Procedure II* (Method of Perlzweig *et al* slightly modified)

Twenty ml of concentrated HCl and 0.5 ml of concentrated HNO<sub>3</sub> were added to 20 ml aliquots of the purified urine extracts in 200-ml Kjeldahl flasks The mixture was kept slowly boiling for 1 hour, excessive evaporation being prevented by using a funnel It was then cooled, diluted to about 100 ml and shaken up successively with two lots (30 ml each) of ether to remove ether-soluble pigments The mixed ethereal layer was washed once with 30 ml of water The washings were added to the main bulk of the extract The ether present dissolved in the aqueous extract was expelled by warming to 60°C The nicotinic acid in the extract was adsorbed on two 1.5-g lots of Fuller's earth by shaking each time for 5 minutes The activated earth was separated as usual on the centrifuge and washed once with 50 ml of N/10 HCl It was then taken up in 50 ml of N/5 barium hydroxide, the mixture heated in a water-bath for 10 minutes, cooled and centrifuged The eluate was separated and the residue in the centrifuge tube was re-eluted in the above manner using 30 ml of N/5 barium hydroxide After removing the barium present in the eluate as the sulphate, the solution was adjusted to pH 7, filtered and made up to volume (100 ml to 200 ml depending on the amount of nicotinic acid present) The final extracts were less intensely coloured than those obtained by using procedure I The nicotinic acid in the extracts was estimated using aqueous aniline and metol, according to the colorimetric procedures of the author and Perlzweig *et al* respectively The results obtained by applying the two different procedures to the same samples of urine are shown in Table I —

TABLE I

*Comparison of values given by the methods of Swaminathan (1939) (A) and of Perlzweig et al (1940) (B)*

Urine sample (representing daily excretion of one rabbit) number	Amine used	A (Procedure I) ( $\mu$ g nicotinic acid)	B (Procedure II) ( $\mu$ g nicotinic acid)
1	Aq aniline	135	128
	Metol	Deep brown interfering colour	122
2	Aq aniline	126	122
	Metol	Deep brown interfering colour	116

TABLE I—*concl'd*

Urine sample (representing daily excretion of one rabbit) number	Amine used	A (Procedure I) ( $\mu$ g nicotinic acid)	B (Procedure II) ( $\mu$ g nicotinic acid)
3	Aq aniline	145	428
	Metol	Deep brown colour	436
4	Aq aniline ..	582	564
	Metol .	Deep-brown colour	556

Table I shows that when aniline was used as the aromatic amine, the values obtained by following the two procedures were the same. When metol was used in the extracts obtained according to procedure II, the values were similar to those obtained with aniline. Addition of metol to the urine extracts prepared according to procedure I resulted in the formation of deep-brown interfering colours and colorimetric comparison was not possible.

*Effect of using different decolorizing agents and the residual colour present in the extract on the nicotinic acid values*—The investigations described below brought out the following points: (1) that charcoal and other decolorizing agents do not adsorb nicotinic acid from urine extracts, (2) the residual colour present in the extracts does not interfere when it is allowed for as a blank under the conditions described by the author, and (3) side reactions of the aromatic amines take place only when the test is performed using the amine hydrochlorides or sulphites in acid medium.

Twenty-ml samples of concentrated urine equivalent to the daily output of individual rabbits receiving nicotinic acid were hydrolysed according to procedure I. Decolorization was effected using the following reagents: (1) charcoal 'Norit', (2) charcoal 'Darco', (3) zinc hydroxide, (4) lead hydroxide, and (5) by adsorption of the nicotinic acid on Fuller's earth and elution with barium hydroxide as under procedure II. The final solutions were adjusted to pH 7 and each made up to 200 ml. Ten-ml aliquots were used for the estimation of nicotinic acid using aqueous aniline according to the author's procedure. The results are shown in Table II.

Similar values were obtained for the nicotinic acid content of the same sample, irrespective of the decolorizing agent employed and the amount of residual colour present, when the estimations were carried out in neutral aqueous medium using aqueous aniline.

TABLE II

*Effect of using different decolorizing agents and the presence of residual colour on nicotinic acid values*

Decolorizing agent employed	Solution	Reading of the standard 20 $\mu$ g nicotinic acid, S D	Reading of the unknown, S D	Strength of unknown, $\mu$ g	Nicotinic acid found in 10 ml, $\mu$ g	Nicotinic acid excretion per rabbit daily, $\mu$ g
Charcoal Norit	Test	40	22.2	36.1		
	Aniline blank	20	37.6	10.7	25.4	508
	Dilution blank	20	37.8	10.6	25.5	510
Charcoal Darco	Test	40	21.4	37.4		
	Aniline blank	20	33.3	12.1	25.3	506
	Dilution blank	20	33.6	11.9	25.5	510
Zinc hydroxide at pH 9	Test	40	23.8	33.6		
	Aniline blank	20	43.5	9.2	24.4	488
	Dilution blank	20	43.9	9.1	24.5	490
Lead hydroxide at pH 8	Test	40	23.4	33.8		
	Aniline blank	20	42.9	9.3	24.5	490
	Dilution blank	20	43.2	9.3	24.5	490
Adsorption on Fuller's earth and elution with $\text{Ba}(\text{OH})_2$	Test	40	26.5	30.2		
	Aniline blank	10	41.2	4.9	25.3	506
	Dilution blank	10	41.8	4.8	25.4	508

S D = Scale divisions

*Effect of using different amines under the conditions described by different workers on the nicotinic acid content of urine*—Aniline,  $\beta$ -naphthylamine, metol (p-methylaminophenol sulphate) and p-aminoacetophenone have been used as the aromatic amine in the cyanogen bromide reaction by different workers (Swaminathan, 1938, Shaw and Macdonald, 1938, Melnick and Field, *loc cit*, von Euler, Schlenk,



Heiwinkel and Hogberg, 1938, Bandier and Hald, 1939, Perlzweig *et al*, *loc cit*, Dann and Handler, 1941) Metol has been preferred to other aromatic amines by certain workers, as it gives a colour which is stable for long periods in the dark. But it has the disadvantage of being an unstable amine, easily oxidized to coloured substances by exposure to light or by mild oxidizing agents. In the present investigation, it was found that monomethylaniline, containing the same secondary amino group as metol, gives a colour which is stable for long periods and is less easily affected by light. It is interesting to note that the two secondary amines give stable colours, while the colours obtained with the primary aromatic amines, viz aniline,  $\beta$ -naphthylamine, and p-aminoacetophenone, are unstable. Aniline has the advantage over other amines that it can be used as an aqueous solution in neutral medium, when the side reactions of the aromatic amines do not take place. The relative intensity and stability of the colours obtained with different amines under the conditions described by different workers are shown in the Graph. The values obtained for the nicotinic acid content of the same urine sample, using different amines according to different workers, are given in Table III.

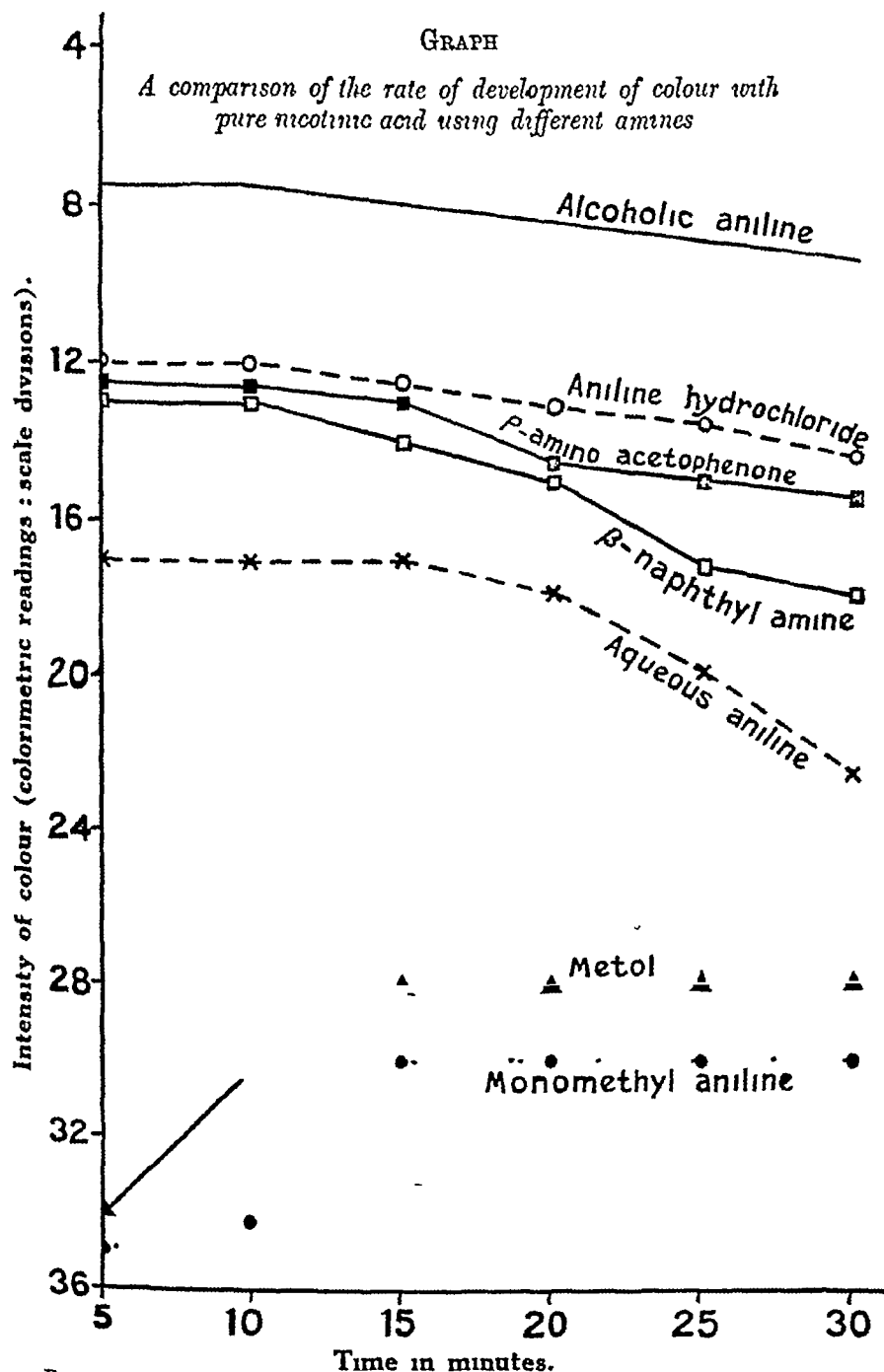
Table III shows that when aqueous aniline is employed the values are the same irrespective of the method of hydrolysis and decolorization, and the 'amine blank' is always the same as the 'dilution blank'. But when the amine hydrochloride or sulphate is employed, the value of the 'amine blank' is always greater than the 'dilution blank', showing that substances are present in urine extracts which react to form coloured complexes with the aromatic amines in acid medium. These are to a great extent eliminated when the adsorption procedure is followed. Even with extracts obtained by following the adsorption procedure, the value of the 'amine blank' is appreciably greater than that of the 'dilution blank'. Under these conditions, the nicotinic acid content of the urine sample under examination cannot be accurately calculated, as the side reactions of the aromatic amines complicate the value of the blanks. The results support the conclusion reached before by the author (Swaminathan, 1941) that reliable results can most consistently be obtained when the test is performed in *neutral aqueous medium using aqueous aniline*.

*Estimation of nicotinic acid in the experimental diet and faeces* — Since the amount of nicotinic acid present in the basal diet was too small for direct determination, the adsorption method described under procedure II for urine was applied to extracts of the diet prepared as described below —

Two hundred g of the finely powdered diet was extracted thrice with hot 1 per cent acetic acid instead of water as described before (Swaminathan, 1938), 600 ml of the solvent being used for each extraction. The mixed extracts were evaporated to about 200 ml on a water-bath. The protein derivatives were removed by precipitation with basic lead acetate and the excess of lead removed as sulphate. The solution was then adjusted to pH 5 and evaporated to about 100 ml on a water-bath. One-fourth of the volume of concentrated HCl was then added and the mixture heated in a water-bath for one hour to hydrolyse nicotinic acid derivatives to nicotinic acid.

GRAPH

*A comparison of the rate of development of colour with pure nicotinic acid using different amines*



Forty micrograms of standard nicotinic acid were treated with the respective reagents under the conditions described in the text and the colours obtained were compared against an artificial standard of 0.1 per cent potassium ferricyanide set at 10 scale divisions

TABLE III

The estimation of nicotine acid in the urine of rabbits using different amines according to different workers

Amine used	Solution	URINE TREATED ACCORDING TO PROCEDURE I					URINE TREATED ACCORDING TO PROCEDURE II				
		COLORIMETRIC READINGS		Strength of the unknown nicotinic acid, $\mu\text{g}$	Nicotinic acid found in 10 c.c. extract, $\mu\text{g}$	Nicotinic acid excretion per rabbit daily, $\mu\text{g}$	COLORIMETRIC READINGS		Strength of the un- known, $\mu\text{g}$	Nicotinic acid found in 10 ml, $\mu\text{g}$	Nicotinic acid excretion per rabbit daily, $\mu\text{g}$
		Reading of the standard 20 $\mu\text{g}$ , S D	Reading of the unknown, S D				Reading of the standard 20 $\mu\text{g}$ , S D	Reading of the unknown, S D			
1	2	3	4	5	6	7	8	9	10	11	12
Aqueous aniline (Swamina- than, 1939)	Test Amine blank Dilution blank	40	22.8	35.0	24.6	452	40	28.1	28.5	23.6	472
		20	38.2	10.4	24.6	452	10	40.5	4.9	23.5	470
		20	38.8	10.4			10	41.6	4.8		
Aniline hydrochloride (Swaminathan, 1941)	Test Amine blank Dilution blank	40	24.1	33.2	14.4	288	40	26.4	30.3	20.5	410
		20	20.2	19.8	21.9	438	10	20.5	9.8	23.2	464
		20	35.4	11.3			10	28.2	7.1		
P-aminacetophenone hydrochloride (Harris and Raymond, 1939)	Test Amine blank Dilution blank	40	22.4	37.2	13.7	264	40	26.2	30.6	18.5	370
		20	17.8	22.5	24.8	456	10	16.6	12.1	22.5	450
		20	32.3	12.4			10	25.2	7.9		
Metol (p methylaminophenol sulphate) (Bandler and Hald, 1939)	Test Amine blank Dilution blank	40	(Solution coloured deep-brown) (Solution coloured deep brown)				40	22.2	36.0	20.2	404
		20					20	25.4	15.8	23.7	474
		20	23.5				20	32.5	12.3		
Monomethylamine hydro- chloride (Swaminathan, present investigation)	Test Amine blank Dilution blank	40	18.8	43.7	18.8	376	40	23.4	34.2	18.1	362
		20	16.3	24.5	27.8	556	20	24.8	16.1	23.9	478
		20	25.2	15.9			20	39.2	10.3		

S D = Scale divisions

Note—Monomethylamine was used in the same concentration as metol. The 'test' contained 10 c.c. of the unknown solution to be tested, together with the requisite amounts of cyanogen bromide and the amine solution. The amine blank was prepared in the same manner as the 'test', except that water was added instead of CNBr. The 'dilution blank' contained water instead of CNBr and the same quantity of acid as that present in the amine solution.

The mixture was then neutralized and adjusted to pH 1 to 2. The nicotinic acid present was adsorbed on Fuller's earth and eluted with barium hydroxide and estimated by the method adopted under procedure II for urine.

*Fæces*—The fæces were extracted in the same way as the diet, the rest of the procedure being as described under procedure II for urine.

*Tissues*—The simplified procedure recently described by the author for foods was followed (Swaminathan 1942).

## SECTION B

### *Estimation of trigonelline in urine, fæces and food*

Recently, two methods have been described for the estimation of trigonelline in biological materials (Sarett, Perlzweig and Levy 1940, Melnick, Robinson and Field, 1940, Kodicek and Wang, 1941). The method used by Sarett and his co-workers and Melnick *et al* involves the conversion of trigonelline to nicotinic acid in the presence of strong alkali and ammonia. According to the former group of workers the percentage of trigonelline added to urine converted to nicotinic acid is 65 to 75, while the corresponding figure put forward by the latter group is 28 to 38. The method of Kodicek and Wang (*loc cit*) is based on the observation that when trigonelline is hydrolysed with alkali in alcoholic solutions, methylamine is split off leaving the ring open. The resulting product combines with benzidine hydrochloride giving an orange-red colour suitable for colorimetric estimation.

In the present investigation an attempt was made to determine the trigonelline present in rabbit urine and fæces following the above two methods. It was found that the recovery of trigonelline added to rabbit urine was of the order of 30 to 45 per cent when the method of Sarett *et al* with some slight modifications was followed. The recovery of trigonelline added to human urine varied from 50 to 70 per cent. The low recovery could not be improved by changing the conditions of hydrolysis and the concentration of urea and ammonium salts. Reliable results could not be obtained using the Klett colorimeter when the method of Kodicek and Wang (*loc cit*) was applied to rabbit urine and extracts of fæces, as certain interfering substances present produced an yellow to yellow-brown colour under the same conditions, interfering in the colorimetric estimation of trigonelline. Attempts to remove these interfering substances have not so far proved successful. Hence, the method of Sarett *et al* (*loc cit*) was adopted for the determination of trigonelline in urine, fæces and food.

*Procedure*—Urine was purified and hydrolysed with 6 N HCl HNO<sub>3</sub> mixture as described under procedure II for the determination of nicotinic acid. Fæces and diet were extracted and the extract hydrolysed as described before for the nicotinic acid estimations. The solutions were then adjusted to pH 10, evaporated if necessary on a water-bath to a small bulk and made up to volume so that 10 ml of the final solution represented the daily urinary or faecal output of one rabbit.

Ten-ml aliquots of the acid-hydrolysed solution were taken in two test-tubes (50 ml capacity). One g of urea and 10 ml of 12 N NaOH were then added to

each tube One ml of a standard solution containing 100  $\mu\text{g}$  of pure trigonelline was added to one of the test-tubes to find out the recovery of added trigonelline The contents of the test-tubes were well mixed by means of a glass-rod, and the mixture heated in a water-bath maintained at 75°C to 80°C for 45 minutes The solutions were then transferred into small beakers and were adjusted to pH 1.2 by the careful addition of concentrated HCl The subsequent manipulations were the same as those described under procedure II for the determination of nicotinic acid The nicotinic acid values obtained in this analysis include the nicotinic acid present in 10 ml of the original acid-hydrolysed extract plus the nicotinic acid formed from trigonelline after hydrolysis with 6 N NaOH and urea Nicotinic acid present in 10 c.c. of the original extract was determined separately and the nicotinic acid formed from the trigonelline by treatment with 6 N NaOH and urea was obtained by difference The recovery of added trigonelline ranged from 35 to 45 per cent Hence, all the values obtained for the trigonelline content were corrected using the corresponding recovery values The values were finally multiplied by the factor 1.11 which represents the ratio of the molecular weights of trigonelline (137) and nicotinic acid (123)

Trigonelline present =

$$\frac{1.11 \times 100 \times \text{Nicotinic acid formed from trigonelline by alkali urea treatment}}{\text{Per cent recovery of trigonelline added to the same}}$$

The calculations are illustrated in Table IV —

TABLE IV.

*The trigonelline content of rabbit urine and the recovery of added trigonelline*

(Figures represent the daily excretion per rabbit)

Urine sample number	NICOTINIC ACID FOUND		Nicotinic acid formed from trigonelline, $\mu\text{g}$	Recovery of added trigonelline, per cent	Trigonelline present, after correction for recovery, $\mu\text{g}$
	After hydrolysis with 7 N HCl, $\mu\text{g}$	After hydrolysis with 7 N HCl, followed by 6 N NaOH and urea, $\mu\text{g}$			
1	120	156	36	35	114
2	125	162	37	40	103
3	150	178	28	42	74
4	625	716	90	39	256
5	465	525	60	38	175
6	468	535	67	43	174

## SECTION C

*Experimental diets* — Considerable difficulty was experienced in devising a diet with a low nicotinic acid content, reasonably adequate as regards its content of other food factors, and at the same time acceptable to rabbits. The normal diet of rabbits contains large amounts of cellulose and they will not take a synthetic diet based on purified protein, starch and fat of the type usually given to rats in vitamin experiments. To overcome the difficulty of providing cellulose, McCay (1929) suggested the use of regenerated cellulose, while Passmore (1935) used the commercial product 'Diaphene' as a source of roughage.

The diets used in these experiments were partially 'synthetic', but the main ingredient was extracted oats, which contains abundant cellulose. The other ingredients were sugar, purified casein, whole milk and a salt mixture. Milk was included because it is a good source of the B<sub>2</sub> group of vitamins with the exception of nicotinic acid. The composition of the diets was as follows (Table V) —

TABLE V  
*The composition of the diets*

Ingredients	DIET I	DIET II
	Parts	Parts
Extracted oats	560	460
Sugar	100	100
Casein (purified)		100
Milk, whole, cow's	310	310
Salt mixture	30	30

The extracted oats was prepared as follows. Crushed oats (2 lb) was added to water 10 litres and the mixture heated to boiling with continuous stirring. The boiling was continued for about 30 minutes. The mixture was cooled and the oats separated by straining the water through a sieve. It was then washed on the sieve 4 to 5 times with cold water, using about 3 litres of water for each washing. The moist oats was squeezed out well and dried in the sun.

The mixed diets were periodically analysed for nicotinic acid and found to contain on the average 70  $\mu$ g and 82  $\mu$ g per 100 g respectively, i.e. their content

of nicotinic acid was low. Diet II differed from diet I only in the fact that 100 parts of purified casein replaced 100 parts of extracted oats. The animals consumed the diets readily.

*Metabolism experiments*—Two groups of 5 rabbits, with an average weight of about 1,200 g, were fed on diet I for a period of 6 weeks, and a diet II for an additional period of 4 weeks. Three animals from each group (Nos 1, 2 and 3 from group I and Nos 6, 7 and 8 from group II) were placed individually in metabolism cages. Each animal in group II received daily in addition 3 mg of nicotinic acid given orally. All the animals were given 0.5 ml of shark-liver oil twice weekly to provide vitamin A. This amount supplied about 2,000 International units of vitamin A. The animals were given a weighed amount of the diet and the food residue was collected and weighed to determine the food intake. The intake of nicotinic acid was calculated from the food intake.

*Collection of urine and faeces*—Urine and faeces were collected daily. To prevent decomposition of urine, 10 ml of 5 per cent acetic acid and 0.5 ml of toluene were placed in each flask used for the collection of urine. The metabolism cages were washed daily with small quantities of water and the washings added to the urine. Urine and faeces were preserved in a refrigerator. Nicotinic acid and trigonelline determinations were made on the weekly collections. The animals were weighed weekly.

The metabolism data are given in Table VI. Data regarding the nicotinic acid content of liver and muscle and the body-weights of rabbits are shown in Tables VII and VIII respectively.

*Intake and excretion of nicotinic acid and trigonelline*—The daily intake of nicotinic acid in rabbits fed on the diets low in nicotinic acid was on the average 97 micrograms (Table VI), the intake of trigonelline being *nil*. The total excretion of nicotinic acid and trigonelline per rabbit in the urine and faeces was on the average 282  $\mu$ g and 169  $\mu$ g in the same animals. Excretion thus considerably exceeded intake. The animals receiving 3 mg of nicotinic acid daily excreted 578  $\mu$ g and 136  $\mu$ g of nicotinic acid in urine and faeces respectively, the corresponding excretion of trigonelline was 184  $\mu$ g and 89  $\mu$ g. Hence about 20 per cent of the ingested nicotinic acid appeared in the urine, the faecal excretion being of the same order as that of the group not receiving nicotinic acid. The fate of the large portion of the ingested nicotinic acid not excreted in the form of nicotinic acid or trigonelline is a problem for further investigation.

*Nicotinic acid content of tissues*—Only two kinds of tissue, liver and muscle, were analysed. The nicotinic acid content of the liver and muscle of animals fed on the unsupplemented diets (Table VII) was on the average 13.5 mg and 7.5 mg per 100 g respectively. The corresponding values for the tissues of animals receiving extra nicotinic acid were 13.8 mg and 7.7 mg per 100 g respectively, being approximately the same as those obtained in the case of animals not receiving nicotinic acid.

TABLE VI

Daily average intake and excretion of nicotinic acid and trigonelline in rabbits on (I) the diet low in nicotinic acid and (II) the same diet supplemented with nicotinic acid  
(Micrograms per rabbit)

GROUP I										GROUP II					
Period (weeks)	Food intake, g	INTAKE OF		URINARY EXCRE TION OF		FÆCAL EXCRE TION OF		Food intake, g	INTAKE OF		URINARY EXCRE TION OF		FÆCAL EXCRE TION OF		
		Nicotinic acid, µg	Trigonelline, µg	Nicotinic acid, µg	Trigonelline, µg	Nicotinic acid, µg	Trigonelline, µg		Nicotinic acid, µg	Trigonelline, µg	Nicotinic acid, µg	Trigonelline, µg			
1	125	88	Nil	120	85	165	80	128	3090	Nil	516	207	128	75	
2	132	92	"	132	93	128	95	135	3095	"	485	180	142	106	
3	120	84	"	153	76	126	75	125	3088	"	695	155	120	106	
4	115	102	"	145	84	116	73	140	3098	"	586	168	136	96	
5	135	95	"	136	65	132	90	132	3092	"	635	175	153	80	
6	140	98	"	153	77	145	85	124	3087	"	589	145	146	98	
7	120	98	"	148	82	136	65	126	3103	"	656	185	130	92	
8	125	103	"	164	95	144	88	122	3100	"	560	208	142	96	
9	130	107	"	165	108	134	92	131	3107	"	525	218	138	85	
10	128	105	"	156	93	128	84	133	3109	"	538	196	125	78	
AVERAGE		97		147	86	135	83		3097		578	184	136	89	



TABLE VII  
*Nicotinic acid content of liver and muscle*

Rabbit number	Diet	Weight of liver, g	Liver, mg /100 g	Muscle, mg /100 g
1	Basal diet alone	64.3	13.1	6.8
2	"	71.1	13.4	7.3
3	"	67.2	13.5	7.8
4	"	65.5	13.2	7.6
5	"	66.5	14.3	8.1
	Average	66.3	13.5	7.5
6	Basal diet + 3 mg nicotinic acid	64.5	13.3	7.1
7	"	72.5	13.8	7.5
8	"	63.8	14.0	8.0
9	"	62.1	14.2	8.1
10	"	60.2	13.9	7.8
	Average	64.6	13.8	7.7

TABLE VIII.  
*Initial and final weight of rabbits (in 10 weeks period).*

Rabbit number	Diet	Sex	Initial body weight, g	Final body-weight, g	Difference in body weight, g
1	Basal diet alone	Male	1,310	1,525	215
2	"	Male	1,300	1,605	305
3	"	Male	1,370	1,540	170
4	"	Male	1,050	1,345	295
5	"	Female	1,380	1,570	190
		Average	1,282	1,517	235

TABLE VIII—*concl'd*

Rabbit number	Diet	Sex	Initial body weight, g	Final body weight, g	Difference in body weight, g
6	Basal diet + 3 mg nicotinic acid	Male	1,350	1,520	170
7	"	Male	1,200	1,425	135
8	"	Male	1,220	1,450	230
9	"	Female	1,170	1,405	235
10	"	Female	1,120	1,225	105
		Average	1,230	1,405	175

*Changes in body-weight*—Table VIII shows the initial and final body-weights of the animals in the two groups. All the animals showed increase in weight during the experimental period. Actually the animals on the unsupplemented diet put on somewhat more weight than those receiving the nicotinic acid supplement, but the difference is probably not significant. There was no evidence of the development of pathological conditions in any of the animals. All remained in good condition.

#### DISCUSSION

The results show that rabbits do not require an extraneous source of nicotinic acid and that they are able to synthesize it. Similar observations have been made before in rats and sheep (Shourie and Swaminathan, *loc cit*, Dann, *loc cit*, Winegar *et al*, *loc cit*). Recently, Pearson and Winegar (1940) have reported studies on the metabolism of nicotinic acid in rabbits fed on a pellagra-producing diet (based on maize) and a normal stock diet. Passmore (*loc cit*) has reported that rabbits can thrive well on diets devoid of vitamin B<sub>1</sub>.

It is possible that only a part of the nicotinic acid contained in the faeces was derived from ingested food, the other part being produced by micro-organisms, a process analogous to the synthesis of other B vitamins in the rumen (McElroy and Goss, 1939).

#### SUMMARY

1. An investigation of the cyanogen bromide method as applied to urine has been made. Special attention has been paid to the effect on nicotinic acid values of using various decolorizing agents, the presence of residual colour and the use of different aromatic amines under different conditions. It has been shown that consistent results can be obtained by carrying out the colour reaction in a neutral aqueous medium using aqueous aniline and the use of different decolorizing agents and the presence of residual colour does not affect the values under such conditions.

2 Nicotinic acid balance experiments were carried out on two groups of rabbits. Both groups were fed on a diet low in nicotinic acid (70  $\mu\text{g}$  per 100 g) for a period of 10 weeks. The animals in the second group received in addition 3 mg of nicotinic acid daily.

3 The average daily intake of nicotinic acid in the animals on the unsupplemented diet was about 97  $\mu\text{g}$ , while the daily average urinary and faecal excretion of nicotinic acid was 147  $\mu\text{g}$  and 135  $\mu\text{g}$  respectively, the excretion of trigonelline being 86  $\mu\text{g}$  and 83  $\mu\text{g}$ . The total excretion was 4 to 5 times the intake. The animals which received extra nicotinic acid excreted about 15 to 20 per cent of the intake in the urine, the faecal excretion being approximately similar in both groups.

4 There was no appreciable difference in the nicotinic acid content of the liver and muscle of the animals fed on the supplemented and unsupplemented diets. All the animals remained in good health and put on weight, irrespective of the nicotinic acid intake.

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## VITAMIN A CONTENT OF LIVER OILS OF SOME INDIAN ELASMOBRANCHS

BY

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[Received for publication, August 15, 1942]

EXTRACTION of oil from shark livers in India is no new question. Work on the subject is indeed old, having been done for a number of years, but the question has been brought to the forefront by the stoppage of supplies of cod-liver oil as the result of the war. Quite a number of provinces in India have been engaged in the extraction of oil from shark livers, but the credit for sustained work on the problem, as well as its extraction on a commercial scale, goes to the Madras Fisheries Department, which is undoubtedly the pioneer in the field. Extraction of oil from shark and saw-fish livers was undertaken by the Department of Fisheries, Madras, in 1934 and the results of its work were set forth in its annual reports.

Extraction of oil both in Bombay and Bengal was originally, for experimental purposes, to obtain scientific data, the object being primarily scientific, viz to gauge the yield of oil from livers of various fishes and to determine their different vitamin A content. Attention in Bengal was concentrated mainly on fresh-water fishes, such as 'rôhit', 'kâtlâ', 'mrigal', 'chital', 'shillang', etc. Extraction of oil from the livers of some of the marine *teleostean* fishes of Bombay, as also an analysis of the vitamin A content of these oils, was undertaken by Niyogi *et al* (1941), who employed the ether process for the extraction of oil. However, the smallness of livers of fishes used by them, the consequent negligible quantities of oils obtained and the costliness of the process of extraction render the extraction of oil from these fishes on a commercial scale an uneconomic proposition. If the extraction of oil on a commercial basis is to be paying, it has to be procurable in gallons by easy and inexpensive methods.

It was with this object in view that the author concentrated his attention on cartilaginous fishes which attain considerable size and are fairly abundant along our coast. Experiments showed that sharks and saw-fishes were undoubtedly the best for the extraction of oil on a commercial scale. These fishes possess

a number of outstanding advantages. Their livers are large, extraction of oil from them is simple and inexpensive, and the yield comparatively copious. Their liver oils are the cheapest source of vitamin A in concentrated form yet found.

Great variation was noticed in the vitamin A content of the liver. The oil of the older sharks was observed to be superior, being richer in vitamin A content. The yield from females which were about to breed was low, but the proportion of vitamin A was higher. Season was also noticed to have a marked effect on the vitamin A content of oils.

Only the results of tests of the vitamin A content of the oil from the livers of sharks, skates and rays have been included in the present paper. There are several connected problems, and such aspects as have not yet been investigated with the fullness they deserve will form the subject of a separate paper. The present paper, accordingly, gives only a synopsis of the results in respect of vitamin A content of oils extracted from the various cartilaginous fishes landed in Bombay.

#### EXPERIMENTAL

*Equipment*—Steel drum used as water-bath, brazier, tinned copper container with lid (on the top of drum), mincing machine, tin trays, aluminium pots, mugs, ladles, enamel buckets, tubs, vats, filter cloth, etc (see Plate XIII).

*Method of extraction*—Fresh livers were thoroughly washed free from adhering dirt and blood and their gall bladders removed. They were minced to a pulpy mass, which was then put into a heavily-tinned copper container with a quantity of warm water. The container was immersed in a hot water-bath to cook the liver mass. The contents of the container were thoroughly stirred from time to time to prevent the liver being overcooked, and care was taken not to raise the temperature of the mass beyond 70°C. Gradually the oil floated to the top and was carefully ladled off and collected separately. The ladling process was continued until no more oil floated to the top. The oil so obtained was filtered free from any liver particles or tissue and then dehydrated with anhydrous sodium sulphate, the dehydration being allowed to continue for 24 hours, after which the clear supernatant oil was decanted off and stored. The oil thus obtained was used for the vitamin A analysis.

*Vitamin A assay*—The assays were carried out on whole oils with the help of a Lovibond tintometer, B D H pattern, the antimony trichloride method of Rosenheim and Drummond (1925) being used, as modified by Carr and Price (1926) and further standardized by Coward *et al* (1931). Artificial light from a white light cabinet was used throughout. Oil solutions were prepared by making 0.5 to a gramme of oil to a known volume, in dry, re-distilled chloroform. If 0.2 c.c. of this solution gave an intense blue coloration with 2 c.c. of a saturated solution of antimony trichloride in chloroform, further dilutions of the original solution were so adjusted as to obtain a final solution 0.2 c.c. of which gave blue values between 4 and 6 on the Lovibond scale. Yellow and neutral glasses were frequently used to match the colour, but were ignored in the calculation of blue values.

PLATE XIII



Apparatus for the extraction of oil



The Table gives the vitamin A values of liver oils extracted from a number of *elasmobranchs* on separate occasions, together with their local and scientific names. The maximum lengths of the fishes noted are also included to give a rough idea of the sizes attained by these fishes. It may perhaps be pointed out that the figures for Carr-Price values indicated in the aforesaid table are likely to be lower than those that would be obtained on the non-saponifiable fractions of the oils by about 16 times (Rajagopal, 1941). A possible reason for the lowering of these values is that, as stated by the same author, the 'inhibitors' present in the whole oil mask the intensity of the blue colour. Another factor which also possibly contributes to the lowering of the blue values of the whole oil is that whereas vitamin A usually occurs in the whole oil in the form of esters, it occurs as free alcohol in the non-saponifiable fractions. Several workers have observed that the esters of vitamin A give a less intense colour with antimony trichloride than with free alcohol, although the biological activity of the former is higher than that of the latter.

The appended statement of vitamin values shows that sharks and rays found in Bombay waters, except for *Carcharias tricuspidatus*, are generally rich in vitamin A content. The shark, *Carcharinus melanopterus*, locally known as 'Khada mushi', is exceedingly rich in vitamin A. It grows to a good size, attaining about 10 feet in length and weighing as much as 1,500 pounds. The maximum recorded weight of liver of an individual of this species was 106 pounds. Such livers generally yield from 50 to 70 per cent of oil. This shark is fairly abundant along the West Coast and the extraction of oil from its liver on a commercial basis would be profitable. The exceptional richness of the liver oil of this fish in respect of vitamin A may be gauged from the fact that one sample gave a Carr-Price value of 1,818 and another 1,229. Such oils can be regarded as concentrates of vitamin A. Normal oil from this shark is of a lemon-yellow colour, whereas exceptionally rich oil is high-coloured, being almost reddish-brown. It will be noted from the statement that a very wide disparity exists in the results of tests of oil from different individuals of *Carcharinus melanopterus*, the highest Carr-Price value obtained being 1,818 and the lowest 350. The wide variation seems to be due, as has already been noted above, to the sex and age of the individual, condition of the gonads, and the season of the year.

The Madras Fisheries Department holds the view that hammerhead sharks (*Cestracion blochii* and *Cestracion zygaena*) are the best in respect of the vitamin A content of their oil. This view conflicts with my observation, as my investigations show that *Carcharinus melanopterus* has the richest vitamin A bearing oil. The Bombay hammerhead shark, *Cestracion zygaena*, no doubt yields oil very rich in vitamin A, but this does not stand comparison with *Carcharinus melanopterus* which has undoubtedly proved itself the prince of *elasmobranchs* in respect of the superlative quality of its oil. *Cestracion zygaena* yields oil very rich in vitamin A, but individuals of this type exceeding six feet have rarely been caught in our waters. Its small liver does not yield more than 30 per cent of its weight of oil, though the latter may be very rich.



TABLE

Serial number	NAME OF FISH		Maximum length noted	Colour of oil	Carr Price values of the oils with dates of extraction	Average Carr-Price value
	Local	Scientific				
1	Khada mushu or Kondaicha (Ratnagiri)	<i>Carcharias melanopterus</i>	10'	Normal lemon-yellow Rarely reddish-brown	633 (28-11-1940)	918
					1,229 (27-9-1941)	
					350 (10-11-1941)	
					1,818 (6-1-1942)	
2	Waghbeer or Waghsheer	<i>Galeocerdo tigrinus</i>	13' 2"	Pale yellow	560 (22-1-1942)	131
					148 (27-3-1941)	
					152 (6-9-1941)	
					94 (23-1-1942)	

3	Nali, Vin, Sondel or Khandero	<i>Pristia perrotteti</i>	18' (including the saw)	Orange	180 (11-10-1940) 184 (30-10-1941) 151 (25-11-1941)	173
4	Kanar or Kan mushi	<i>Cestracion blochi</i>	5'	Brown	139 (8-3-1941) 110 (19-9-1941) 268 (20-10-1941)	172
5	Kanar or Kan mushi	<i>Cestracion zygema</i>	6	Amber yellow	514 (20-2-1942) 624 (7-4-1942)	509
6	Ranja or Pok	<i>Rhynchobatus dyedensis</i>	9'	Pale yellow	59 (28-10-1941) 40 (12-3-1942)	49.5

TABLE—*concd*

Serial number	NAME OF FISH		Maximum length noted	Colour of oil	Carr Price values of the oils with dates of extraction	Average Carr-Price value
	Local	Scientific				
7	Pisori	<i>Carcharinus limbatus</i>	7'	Pale yellow	27 (10-1-1942) 40 (13-4-1942) 15 (24-9-1941) 24 (15-3-1942)	33.5
8	Boladi or Wagh	<i>Rhinoptera javanica</i>	4' across disk	Orange	86 (6-2-1942) 14 (22-12-1941) 0 (14-2-1942) 19 (17-10-1941)	19.5
9	Kirwa	<i>Carcharinus pleurotænia</i>	5'	Lemon yellow		86
10	Sunera	<i>Nebrius ferrugineus</i>	9' 5"	Pale brown		14
11	Wagr	<i>Carcharias tricuspidatus</i>	5'	Almost colourless		0
12	Pakat	<i>Dasybatus uarnak</i>	5' 4" across disk	Pale yellow		19

Similar is the hammerhead, *Cestracion blochii*, the oil of which, though fairly good in vitamin A, cannot be regarded as very rich. A variety of shark known as tiger shark or Waghbeer, *Galeocerdo tigrinus*, also attains a considerable size, reaching 13 feet in length and weighing nearly a ton. Its liver, the largest of which examined weighed 150 pounds, yielded a percentage of oil from 60 to 70 per cent. These oils possess a fair content of vitamin A, but it cannot be considered high.

The liver oils from saw fish, *Pristis perotteti*, are fairly rich in vitamin A and the extraction of oil from them is economical since the fish attains a considerable size and has a large liver.

Livers from the ray *Rhynchobatus djeddensis*, which is recorded as growing to 9 feet in length, are too small to be of much value from a commercial point of view. The same may be said of the sharks *Carcharias limbatus* and *Carcharias pleurotænia* and the ray *Rhinoptera javanica*. The shark *Carcharias tricuspidatus* seems to be an exception among the *selachians* in not possessing any vitamin A in its liver oil, which was almost colourless. Further investigation will be necessary to confirm whether this colourless feature of the oil is a regular specific characteristic or not. Generally, the intensity of the colour of liver oils appears to vary directly with the vitamin A content and this view supports Drummond and Hilditch's (1930) observation that 'a yellow oil is probably richer in vitamin A than one pale in colour' provided precaution to remove all traces of liver tissue and accompanying enzymes is taken.

#### ACKNOWLEDGMENT

The author takes this opportunity to express his obligation to Dr S B Setna, Fisheries Officer, under whom the work was done, for his valuable advice and suggestions at every stage of the investigation.

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## DENTAL CARIES IN CHILDREN IN MADRAS CITY IN RELATION TO ECONOMIC AND NUTRITIONAL STATUS

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[Received for publication, July 1, 1942]

In a previous paper (Shourie, 1941) the incidence of dental caries in 6,866 children in various parts of India was recorded. It was found that Indian children in general show much less caries than children in Northern Europe and America, and that the consumption of diets which are very defective in comparison with generally accepted standards is compatible with relative freedom from caries. The study of dental caries in India has now been extended by the examination of 1,886 children attending schools in Madras city. The investigation was undertaken to throw light on the following points —

- (a) The incidence of caries in a fairly large group of rice-eating children. In the previous investigation the majority of children examined were wheat- or millet-eaters.
- (b) Whether economic status influences the incidence of caries.
- (c) Whether children who are in a poor state of nutrition, as shown by the presence of food deficiency diseases, are more prone to caries than children from such conditions.

### METHOD OF INVESTIGATION.

This was the same as that followed in the earlier survey (Shourie, *loc cit*). A detailed dental inspection, in which all the available tooth surfaces in the mouth were examined, using a dental mirror and probe, was made in the case of each child. The total number of teeth and the number of deciduous and permanent teeth were recorded. All extracted teeth were recorded as carious except the negligible percentage known to have been lost as a result of trauma or extracted

for some other reason. In recording the extent of caries the classification of Day and Sedwick (1934) was employed —

- 1 Initial caries including softened or discoloured pits and fissures giving lodgment to a fine explorer
- 2 Freely accessible approximate cavities and small open cavities involving less than one-fourth of the tooth
- 3 More extensive caries involving more than one-fourth and less than two-thirds of the crown
- 4 Caries involving from two-thirds to complete destruction of the crown

The 'average caries figure' is  $\frac{\text{total caries figure}}{\text{number of teeth examined}}$ . The 'total caries figure' is calculated by multiplying the numbers included under each degree of caries by 1, 2, 3 and 4 as the case may be.

#### GROUPS STUDIED

Five schools were surveyed. These included two girls' schools—a poor and a well-to-do—and 3 boys' schools—a well-to-do, a middle-class and a poor school. It is to be observed that the differences in the social and economic status of the schools were not clear-cut. The well-to-do and middle-class schools included some poor children. In a general way, however, the schools were differentiated as regards the economic status of the children. This is confirmed by the fact that the incidence of deficiency disease was negligible in the well-to-do schools and highest in the poor schools.

*Deficiency diseases*—All children were examined for Bitot's spots, angular stomatitis and phrynoderma. These conditions have been fully described in previous publications of the Laboratories (Aykroyd and Rajagopal, 1936, Aykroyd and Krishnan, 1937). Long experience has shown that their incidence in any group of children provides a reliable index of the state of nutrition of the group. An individual child showing one or other of the signs in question may be regarded as being in a poor state of nutrition. This method of assessing state of nutrition has been used by Wilson and Widdowson (1942) in an extensive survey of Indian children and a full discussion of the subject will be found in their recent *Memoir*.

*Diet*—No diet surveys were carried out in connection with the present investigation, but the Laboratories possess abundant data, collected in numerous surveys, about typical South Indian diets as consumed by families of varying economic status. The relation between diet and the incidence of deficiency diseases has also been extensively studied (e.g. Aykroyd and Krishnan, *loc cit*). These diseases occur most commonly in individuals living on a diet composed mainly of rice and containing few supplementary foods. It may be assumed that the diet of the poor children was in general of this nature. The main difference between the diet of children of superior economic status and that of poor children is that the former includes foods other than rice—milk, pulses, vegetables and fruits—in greater quantities.

TABLE I  
Groups studied

Name of school	Number of children	Economic status	Percentage of children showing deficiency disease	Percentage free from caries
Girls' school A	136	Well to do	Nil	34.5
Girls' school B	338	Poor	6.2	44.9
Boys' school A	527	Well to do	0.09	40.4
Boys' school B	572	Middle class	8.7	38.1
Boys' school C	313	Poor	16.2	34.1
TOTAL	1,886		All children	39.8
Delhi schools (boys and girls)	1,074		" "	39.7

### RESULTS

Table I shows the percentage of children free from caries in the various schools and the percentage incidence of deficiency diseases. The percentage free from caries in another urban area (Delhi) is also given. No consistent differences in the caries incidence are brought out by this rough and ready method of comparison. The percentage free from caries was somewhat higher in the well-to-do and middle-class boys than in the poor boys, but the girls showed a tendency in the opposite direction. The average figure was similar to that given by a group of mainly wheat-eating urban children in North India. The previous investigation (Shourie, *loc cit*) suggested that the incidence of caries is lower in rural than in urban areas. No large group of rural rice-eating children has as yet been surveyed but the Madras rice-eating children definitely showed more caries than rural wheat-eating children. The percentage free from caries in a wheat-eating rural area was found to be 54 (Shourie, *loc cit*).

Tables II, III, IV and V provide details about the incidence of caries in deciduous and permanent teeth in the various age groups. The extent of caries according to the classification previously described is also given. The significance of differences between the incidence in the various age groups in schools of different economic status and in children with and without deficiency diseases was determined.  $X^2$  was calculated by the help of a fourfold table,  $P = 0.05$  being taken as the conventional level of significance (Bradford Hill, 1937).



TABLE II  
Incidence of dental caries in poor and well-to-do children

(Girls)

Age in years	Poor (P) or well-to do (W)	Number of children	Percentage free from caries	Whether significant or not	DECIDUOUS TEETH		Difference whether significant or not	PERMANENT TEETH		Difference whether significant or not	All teeth Percentage carious
					Number	Percentage carious		Number	Percentage carious		
7	P	12	33.3	N	190	16.3	N	90	4.4	N	12.5
	W	3	33.3		39	10.2		33	0.0		5.5
8	P	14	28.5	N	199	20.6	N	137	5.1	N	14.2
	W	2	50.0		25	20.0		23	8.7		14.5
9	P	10	20.0	N	108	27.7	N	132	3.7	N	14.5
	W	21	23.8		217	23.5		291	3.4		12.0
10	P	17	47.0	N	133	11.2	S	286	2.1	S	5.0
	W	17	23.5		128	21.1		289	4.8		9.8
11	P	36	44.4	N	136	29.4	S	799	3.8	N	7.5
	W	20	45.0		91	10.9		411	4.3		5.5
12	P	38	50.0	N	35	17.1	N	1,000	3.8	N	4.2
	W	15	40.0		25	32.0		379	5.2		6.9
13	P	33	57.5	N	9		N	909	2.8	S	3.3
	W	13	38.4		2			356	5.6		5.8

11	P	112	11	5	917	13	N	11
	W	333	9		219	62		61
15	P	500	28		784	51	N	51
	W	342	14		300	68		68
16	P	459	37		1,051	53	S	53
	W	555	9	3	252	19		23
17	P	518	27		781	11	N	11
	W	333	0	3	165	48		53
18	P	533	15	1	110	50	S	52
	W	160	6		173	156		156
19	P	343	14		402	67	N	67
	W	00	1		30	100		100
20	P	625	8		236	38		38
	W		0					

	Total number of teeth	Percentage of teeth carious	Extent of caries				Average caries figure
			1	2	3	4	
Deciduous	Poor	20.9	72	95	32	33	0.44
	Well to do	18.3	38	42	21	7	0.4
Permanent	Poor	4.4	210	64	43	42	0.07
	Well to do	5.5	117	28	16	9	0.08

TABLE III  
Incidence of dental caries in poor and well-to-do children  
(Boys)

Age in years	Poor (P) or well-to do (W)	Number of children.	Percentage free from caries	Whether significant or not	DECIDUOUS TEETH		Difference whether significant or not	PERMANENT TEETH		Difference whether significant or not	All teeth Percentage carious
					Number	Percentage carious		Number	Percentage carious		
7	P	5	20.0		84	8.3		36	5.5		7.5
	W										
8	P	11	27.2		146	17.8		118	5.9		12.5
	W	3			35	25.7		41	4.8		14.5
9	P	14	21.4		124	28.2		223	9.4		16.1
	W	16	37.5		135	25.9		256	4.6		12.0
10	P	23	21.7		219	26.5		341	4.9		13.5
	W	40	25.0		330	23.0		645	5.6		11.4
11	P	34	23.5		190	33.1		669	5.6		11.7
	W	63	36.5		315	23.1		1,271	4.9		8.5
12	P	22	45.4		86	19.7		473	4.6		6.9
	W	68	45.5		165	26.4		1,629	4.7		6.6
13	P	29	27.6		31	38.7		752	7.2		7.5
	W	96	45.8		51	29.4		2,569	6.1		6.1

11	{ P W }	31	41.0	{ N }	50	3.4	{ S }	{ 708	5.1	{ N }	{ 5.2
		90	40.0		21	33.3			0.5		0.8
15	{ P W }	28	35.7	{ N }	5	80.0	{ N }	{ 725	7.3	{ N }	7.8
		65	43.1		7	28.5		{ 1,841	6.3		0.1
16	{ P W }	39	46.1	{ N }	2		{ N }	{ 1,101	4.9	{ S }	5.1
		44	40.0		1			{ 1,210	7.2		7.2
17	{ P W }	30	40.0	{ N }				{ 855	7.1	{ N }	7.1
		20	50.0					{ 578	1.4		1.4
18	{ P W }	20	50.0	{ N }				{ 590	1.2	{ S }	4.2
		14	35.7					{ 403	8.0		8.0
19	{ P W }	13	30.7	{ N }				{ 387	8.7		8.7
		0	33.3		2			{ 175	6.8		6.6
20	{ P W }	7						{ 218	15.5		15.5
		1						{ 28	10.7		10.7

	Total number of teeth	Percentage of teeth carious	Extent of caries				Average caries figure
			1	2	3	4	
Deciduous	Poor	843	86	47	44	40	0.53
	Well to do	1,052	79	85	46	48	0.55
Permanent	Poor	7,379	330	67	33	20	0.092
	Well to do	13,174	522	132	79	62	0.097

TABLE IV

*Incidence of dental caries in poor and middle-class children**(Boys)*

Age in years	Poor (P) or middle class (M)	Number of children	Percentage free from caries	Whether significant or not	DECIDUOUS TEETH		Difference whether significant or not	PERMANENT TEETH		Difference whether significant or not	All teeth Percentage carious
					Number	Percentage carious		Number	Percentage carious		
7	P	6	20.0	N	84	8.3	N	36	5.5	N	7.5
	M	2	100.0		31	19.3		17	5.9		14.5
8	P	11	27.2	N	146	17.8	N	118	5.9	N	12.5
	M	17	41.2		214	20.5		195	2.0		11.7
9	P	14	21.4	N	124	28.2	N	223	9.4	S	16.1
	M	29	31.0		338	20.4		364	2.7		11.2
10	P	23	21.7	N	219	26.5	N	341	4.9	N	13.5
	M	38	28.9		270	33.6		675	5.6		13.6
11	P	34	23.5	N	190	33.1	N	669	5.6	N	11.7
	M	42	35.7		190	27.8		473	4.6		8.3
12	P	22	45.4	N	86	19.7	N	865	4.1	N	6.9
	M	58	34.5		132	28.9		1,412	0.0		8.0
13	P	29	27.6	N	31	38.7	N	752	7.2	N	7.5
	M	59	37.1		55	49.1		1,578	0.8		8.2

14	P	71	41.0	N	56	3.4	N	709	5.4	N	52
	M	78	48.7		18	22.9		2,117	5.3		57
15	P	28	35.7	N	5			725	7.3	N	78
	M	75	38.6		0			2,114	5.1		57
16	P	39	46.1	N	2			1,104	4.9	N	51
	M	62	40.3		4			1,744	6.4		64
17	P	30	40.0	N				855	7.1	N	71
	M	48	27.1					1,370	5.0		80
18	P	20	50.0	N				590	4.2	N	42
	M	30	40.6					864	4.6		46
19	P	13	30.7	N				397	8.7	S	87
	M	20	40.0					582	5.3		53
20	P	7	0.0	N				218	15.5	S	155
	M	5	60.0					150	2.6		26

	Total number of teeth	Percentage of teeth carious	Extent of caries				Average caries figure
			1	2	3	4	
Deciduous	Poor	23.9	80	47	44	49	0.53
	Middle class	20.7	153	99	43	52	0.53
Permanent	Poor	6.2	330	67	33	29	0.032
	Middle class	5.7	599	125	47	40	0.081

TABLE V

Incidence of dental caries in children showing and not showing signs of deficiency disease

Age in years	Children showing (SS) and not showing (NS) signs	Number of children	Percentage free from caries	Whether significant or not	DECIDUOUS TEETH		Difference whether significant or not	PERMANENT TEETH		Difference whether significant or not	All teeth Percentage carious
					Number	Percentage carious		Number	Percentage carious		
7	NS										
	SS										
8	NS	28	35.7	N	363	16.8	S	314	2.5	N	10.6
	SS	3	100.0		32	50.2		40	5.0		27.7
9	NS										
	SS										
10	NS	94	25.5	N	764	28.0	N	1,542	5.5	N	13.0
	SS	7	28.5		55	20.0		119	4.1		9.1
11	NS	127	33.8	N	643	26.1	S	2,555	4.9	N	9.1
	SS	12	25.0		52	40.4		250	4.4		10.5
12	NS	138	39.8	N	352	25.8	N	3,267	5.4	N	7.4
	SS	10	50.0		21	28.9		247	3.2		5.2
13	NS	169	39.6	N	121	40.4	N	4,501	6.6	N	7.5
	SS	15	46.6		10	31.2		398	37.4		4.8
14	NS	188	43.6	N	113	16.8	N	5,110	5.9	N	6.2
	SS	11	45.4		12	8.3		284	5.4		5.5

15	{ NS SS	155	40.0	{ N	21			1,128	5.0	{ N	0.2
		13	30.7					252	8.5		8.7
16	{ NS SS	131	41.9	{ N	7			3,700	0.3	{ N	6.5
		14	35.7		1			301	0.1		6.1
17	{ NS SS	91	36.2	{ N				2,611	0.0	{ N	6.0
		7	28.5					201	8.4		8.4
18	{ NS SS	56	44.6	{ N				1,622	5.6	{ N	5.6
		8	50.0					235	3.4		3.4
19	{ NS SS	37	37.8	{ N	2			1,088	0.5	{ N	6.5
		2	0.0					56	10.7		10.7
20	{ NS SS										

	Total number of teeth	Percentage of teeth carious	Extent of caries				Average caries figure
			1	2	3	4	
Deciduous	{ NS	2,386	239	176	90	110	0.58
	{ SS	189	22	20	11	9	0.75
Permanent	{ NS	30,744	1,314	300	148	103	0.09
	{ SS	2,483	104	16	6	8	0.072



Statistical analysis failed to reveal any consistent differences between the various economic groups. The well-to-do girls showed more caries in deciduous teeth at 10 years of age than the poor girls, but this tendency was reversed in the next age group (11). Again, as regards permanent teeth, significant differences were observed in only 3 age groups, one being in favour of the poor children, and the other two in favour of the well-to-do. Among the boys, significant differences were likewise present in only a few age groups, and the same inconsistency as regards economic status was observed. Where the differences were statistically significant according to the criterion applied, the level of significance was low. It can, therefore, be assumed that economic status has little influence on the development of dental caries in Madras school children.

The incidence of caries in deciduous teeth in the Madras children was higher than the average incidence observed in the previous investigation. The percentage of deciduous teeth showing caries in Madras children was 23.7, in the previous survey the corresponding percentage was 10.8. The average caries figures for deciduous teeth were 0.23 (Madras) and 0.50 (previous survey) respectively. In the case of permanent teeth, the average caries figure for Madras children (0.08) was also higher than the figure for all children examined in the previous survey (0.06). The large number of children previously examined (6,866) included only a relatively small percentage of rice-eaters, more than half being rural children consuming wheat or millet. It has previously been pointed out that the percentage of children free from caries was similar in the urban areas of Delhi and Madras. The average caries figures were, however, slightly lower in the mainly wheat-eating Delhi children than in the Madras children, the figures for Delhi being 0.21 and 0.05 respectively, as compared with 0.23 and 0.06 for Madras. The results of the present investigation thus in general confirm the previous conclusions that 'urban children in all age groups show more caries than rural children' and that 'wheat-eaters have a slight advantage over rice-eaters'. It is to be observed that the term 'wheat', as used in this and the previous paper, means whole wheat, and not bread made from refined wheat flour.

The most important point emerging from the present investigation is the lack of correlation between state of nutrition and the incidence of caries. Deficiency diseases were more prevalent in the poor than in the middle-class schools, and intake of 'protective' foods, in India as in all other countries, rises with economic status. Nevertheless, the consumption of a better diet did not affect the caries incidence. Children actually suffering from deficiency diseases, and therefore well below the average as regards state of nutrition, showed no tendency to suffer more from caries than the other children. Bitot's spots and phrynoderma are associated with vitamin A deficiency, and angular stomatitis with riboflavin deficiency. It, therefore, follows that deficiency of these vitamins does not predispose to dental caries.

Osborne (1932) pointed out that the incidence of caries is high in Australia, in spite of the fact that the population in general consumes a diet rich in vitamins. Another Australian writer (Earnshaw, 1936) has, however, presented statistical data in support of the view that caries is evidence of general malnutrition. There has been some tendency within recent years to adopt this view in America, a

tendency which is reflected in propaganda material issued by public health authorities in that country. A collection of American posters and pamphlets concerned with 'better teeth' is available in the Laboratories. These in general teach the lesson that a diet containing abundant milk, vegetables, eggs, butter and fruit will ensure the development of good teeth and prevent their decay. Observations in India suggest that such propaganda, while it is doubtless of value in educating the people about diet, is not likely to prove a satisfactory weapon of attack against dental caries.

The relative freedom from caries of Indian children as compared with children in Northern Europe and America was emphasized in the earlier paper. The survey in Madras extends and confirms this observation, for which any satisfactory theory of the aetiology of dental caries must account.

### SUMMARY

- 1 The incidence of dental caries among 1,886 children in Madras city has been investigated. The percentage free from caries was 39.8.
- 2 Economic status had no influence on the caries incidence.
- 3 Children with deficiency diseases did not show more caries than other children.

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# INVESTIGATIONS INTO THE BIOLOGICAL VALUE OF MILK PROTEINS

## Part II

### BY THE BALANCE-SHEET METHOD\*

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[Received for publication, July 1, 1942]

### INTRODUCTION

IN a previous communication from this laboratory (Mitra and Mittra, 1942) the relative growth-promoting properties of the proteins of buffalo, cow and goat milks were estimated by the 'rat-growth' method. In the present investigation an attempt has been made to estimate the biological value of the protein of all the three milks by the 'balance-sheet' method. The technique of employing white rats for assaying the biological value of different edibles was first elaborated by Mitchell (1924) and later considerably modified by Chick *et al* (1935). This modified technique, with slight alterations, has been followed.

### EXPERIMENTAL.

Six laboratory-bred white rats each weighing 200 g to 250 g were used for each series of observations. The food and excreta of two rats (both males) were pooled together and each pair treated as one unit. Each group of six animals or 3 rat units was put on a nitrogen-free diet for a period of seven days and the excreta

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\* Paper read at the inaugural meeting of the *Physiological Society of India* (Patna Branch) on the 7th March, 1942

collected daily for the last half of the period (4 days) in order to estimate the endogenous nitrogen metabolism by means of analysis of the urine and faeces. The *nitrogen-free diet* consisted of the following ingredients: Pure sugar cubes, 90 g, corn starch, 735 g, coco-nut oil, 130 g, McCollums salt mixture, 50 g, and calcium carbonate, 8 g. The diet could not be made absolutely nitrogen-free as the starch used was found to contain 0.05 per cent of nitrogen. The urine was collected daily in bottles containing 5 c.c. of a 5 per cent solution of phenol and 1 c.c. of a 10 per cent solution of thymol to prevent any loss of free ammonia. The faeces were dried on the water-oven, powdered and stored in a refrigerator. The nitrogen content of aliquot portions of urine and faeces was estimated by the Kjeldahl method. After a period of rest for one week (on the laboratory stock diet) the same groups of animals were placed on experimental or test diets for a period of 8 days. The composition of the test diets with three different kinds of milk at 10 and 15 per cent levels respectively is shown in Table I. Fresh whole milk was added daily to the other comparatively stable ingredients of the food mixture in the way described in the previous communication (Mitra and Mittra, *loc cit*). Along with the nitrogen-free and experimental diets each of the animals was given 3 drops of cod-liver oil and 2 c.c. of a 1 per cent solution of Marmite daily as protection against vitamin deficiency.

TABLE I

*Composition of test diets in grammes*

(Calorific value about 2,500)

Items of food	BUFFALO MILK		COW MILK		GOAT MILK	
	10 per cent level	15 per cent level	10 per cent level	15 per cent level	10 per cent level	15 per cent level
Milk	1,908	2,885*	2,050	3,040	1,667	2,506
Corn starch	137	130	157	65	207	127
Sugar cubes	54	54	54	40	54	54
Coco nut oil	<i>Nil</i>	<i>Nil</i>	17	<i>Nil</i>	25	<i>Nil</i>
Salt mixture	24	24	24	24	24	24
Calcium carbonate	6	6	6	6	6	6

\* Partly skimmed to reduce the fat content from an average of 6.44 to 2.85 per cent

During the last 4 days of the observation period with the different experimental diets the intake of nitrogen and its excretion through the faeces and urine were determined and average intake and output of nitrogen per rat per day calculated (*see*

Table II) The daily excretion of faecal and urinary nitrogen per rat per day during the pre-experimental period on the nitrogen-free diet is also shown in Table II. Owing to the unavoidable presence of nitrogen in the (corn) starch used and also in the Marmite solution, the respective milks were not the sole source of nitrogen in the test diets. The non-milk quota of nitrogen, however, barely exceeded 1 per cent of the total nitrogen supplied in the diet. The influence of non-milk nitrogen in the test diets can be safely ignored.

The formulæ suggested by Chick *et al* (*loc cit*) for calculating the relative digestibility coefficient and relative biological value have been used in arriving at the respective figures (Table II). The details of the formulæ are not given here as they have been described twice (Basu *et al*, 1936, Acharya *et al*, 1942) in this *Journal* within recent years.

TABLE II

*The average intake and output of nitrogen per rat per day in grammes*

Rat unit number	NITROGEN-FREE DIET			EXPERIMENTAL OR TEST DIET			Relative digestibility coefficient	Relative biological value	
	Dry food intake, g	NITROGEN EXCRETED, g		Dry food intake, g	Total N intake, g	NITROGEN EXCRETED, g			
		Faeces.	Urine			Faeces			Urine

10 per cent level

*Buffalo*

1B	13.8	0.0277	0.0283	15.2	0.2769	0.0754	0.1023	82.8	67.7
2B	12.8	0.0217	0.0314	14.8	0.2664	0.0705	0.1071	81.7	65.2
3B	14.2	0.0300	0.0322	15.1	0.2739	0.0809	0.1056	81.4	67.1

*Cow*

1C	9.5	0.0184	0.0320	8.0	0.1376	0.0322	0.0653	90.0	73.1
2C	8.6	0.0213	0.0290	11.3	0.1994	0.0514	0.0600	84.9	81.7
3C	9.9	0.0235	0.0286	9.4	0.1586	0.0394	0.0645	90.0	74.8

TABLE II—*conold*

Rat unit number	NITROGEN-FREE DIET			EXPERIMENTAL OR TEST DIET				Relative digestibility coefficient	Relative biological value
	Dry food intake, g	NITROGEN EXCRETED, g		Dry food intake, g	Total N intake, g	NITROGEN EXCRETED, g			
		Fæces	Urine			Fæces	Urine		

*Goat*

1G	13.5	0.0291	0.0405	9.1	0.1749	0.0443	0.0952	91.3	65.7
2G	13.5	0.0246	0.0448	11.8	0.2413	0.0662	0.0939	82.8	75.4
3G	13.1	0.0269	0.0331	12.6	0.2550	0.0720	0.1126	82.3	62.1

## 15 per cent level

*Buffalo*

4B	14.5	0.0239	0.0363	13.0	0.3271	0.0864	0.1488	80.9	57.5
5B	14.0	0.0185	0.0318	9.3	0.2217	0.0576	0.1191	82.4	52.2
6B	13.9	0.0225	0.0349	10.9	0.2658	0.0654	0.1421	83.9	51.9

*Cow*

4C	10.2	0.0197	0.0298	9.9	0.2976	0.0579	0.1618	87.2	49.1
5C	11.1	0.0230	0.0302	10.3	0.3021	0.0598	0.1538	87.8	53.4
6C	11.9	0.0243	0.0301	10.3	0.3005	0.0885	0.1602	85.4	49.3

*Goat*

4G	11.2	0.0274	0.0386	12.2	0.3325	0.0797	0.1883	84.3	46.6
5G	11.9	0.0250	0.0402	12.5	0.3408	0.0715	0.1918	86.4	48.5
6G	12.9	0.0274	0.0403	12.6	0.3416	0.0794	0.1677	84.8	56.0

## DISCUSSION

Little or no difference could be found in the relative digestibility coefficient figures determined at 10 and 15 per cent levels of intake in the case of each of the milk proteins. In comparing the milk proteins at the same level of intake cow milk proteins gave the highest figure and buffalo milk protein the lowest, with the proteins of goat milk coming in between.

Kind of milk.	RELATIVE DIGESTIBILITY COEFFICIENT		RELATIVE BIOLOGICAL VALUE	
	10 per cent	15 per cent	10 per cent	15 per cent
Buffalo	82.0	82.4	66.7	53.9
Cow	88.3	86.8	76.5	50.6
Goat	85.5	85.2	67.7	50.4

In the case of biological value, however, the 15 per cent level of intake seems to be definitely uneconomical as compared to that of 10 per cent in the case of all the three milks. At a 10 per cent level of intake the protein of cow milk seems to be superior to that of the buffalo or of the goat. Mitchell (*loc cit*) has reported the average biological value of whole milk to be 84.7 at a 10 per cent level of intake as compared to the average figure of 76.5 recorded in the present investigation (though one of the rat units gave as high a figure as 81.7). In the present investigation the comparatively lower figures obtained may have been due to the inferior quality of milk used as compared to the milk used by Mitchell. At the 10 per cent level of intake, the proteins of goat milk gave a slightly higher figure as compared with those of buffalo milk. One of the rat units in the case of goat milk gave the high figure of 75.4, while another gave the low figure of 62.1. In the case of buffalo milk no deviation of such magnitude was observed.

At the 15 per cent level of intake no appreciable difference was observed between the three milks. The results, however, were not tested statistically as with only 4 degrees of freedom the conclusions were not likely to be definite.

## SUMMARY

The digestibility coefficient and biological value of the proteins of buffalo, cow and goat milks were determined by balance-sheet method at 10 and 15 per cent levels of intake. No difference in digestibility was observed between 10 and 15 per cent levels of protein intake, in the case of all the three milks the proteins of cow milk gave the highest figure for digestibility, with those of goat and buffalo milk closely following in that order. Lower figures for biological value were



obtained at the 15 per cent level of intake than at the 10 per cent level in the case of all the three milks. At the 10 per cent level cow milk gave the highest figure and no appreciable difference between the proteins of the other two kinds of milk was observed. At the 15 per cent level, the difference between the three were almost negligible.

#### ACKNOWLEDGMENTS

The authors are obliged to Rai Bahadur Dr B P Mozoomdar, Director of Public Health, Bihar, for his interest in the work and encouragement, and to Mr S Chatterjee of the Bacteriophage Laboratory for kindly looking after the animals.

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## A DIET SURVEY OF NIZAMABAD DISTRICT IN H. E. H. THE NIZAM'S DOMINIONS.

BY

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[Received for publication, September 7, 1942 ]

THE present survey is a part of a public health scheme to find out the dietary habits of people and existing deficiency diseases in Hyderabad State. Knowledge of the composition of diets as regards both quantity and quality is the foundation of the scheme which has as its ultimate object the improvement of the nutrition of the people.

### DIET SURVEY.

Diet surveys were carried out in 147 families, consisting of 784 persons, during the months of November and December 1941. Families of cultivators, tradesmen, agricultural labourers and members of the depressed classes were chosen at random for the survey work.

The families are classified into groups according to their monthly income per man value (Table I).

Groups I and II were poor families working as daily labourers. The families in groups III and IV were small agriculturists, undertaking casual labour and small trade in addition to agriculture, due to their smallness of holdings. Groups V and VI consisted of agriculturists depending on agriculture alone for their livelihood.

TABLE I

*Classification of families*

Group	Number of families	Number of members	Total adult man value	Number of consumption units per family	Monthly income per man value	Average monthly income per family	REMARKS
					Rs a p	Rs a p	
I	25	91	68 8	2 7	1 12 4	4 14 0	Daily labourers
II	43	212	160 4	3 7	2 5 10	8 13 4	„ „
III	50	283	210 3	4 2	3 12 8	15 15 4	Small agriculturists with casual labour
IV	15	57	69 8	4 6	5 10 6	26 5 4	Agriculturist with casual labour and trade
V	9	61	47 8	5 3	7 6 10	40 8 10	Depending on agriculture only
VI	7	50	42 2	6 0	9 10 3	56 2 3	„ „

Investigations of income and expenditure among villagers who do not receive regular wages are more difficult than similar inquiries made in towns or cities. The income figures given should therefore be regarded as approximate. The number of consumption units per family steadily increased with increasing income, a fact of considerable interest and importance. A similar state of affairs has been observed in other parts of India and has been commented on by Aykroyd (1941).

The labourers did not always receive their wages in money but were given a certain quantity of grain also in payment for work.

Intake of proximate principles, minerals and vitamins was calculated from the tables of food values from the Health Bulletin No 23 (Aykroyd, 1941a). The family coefficients for calculating the man value of each family (i.e. the number of consumption units which it contained) were those suggested in Health Bulletin No 23.

## RESULTS OF INVESTIGATIONS

Table II shows the composition of the diets as regards foods —

TABLE II

*Foodstuffs in ounces per consumption unit*

Foodstuffs	Group I	Group II	Group III	Group IV	Group V	Group VI
Rice (home pounded)	20 80	19 90	15 90	15 60	20 60	11 00
Rice (milled)		0 30	0 30	2 90	1 90	8 70
Wheat		0 01		0 80	0 15	2 20
Jawar	1 40	1 70	4 40	4 20	3 90	2 70
Ragi	0 01	0 16	0 10			
Maize	0 90	2 50	3 50	1 60		0 10
Pulses	0 60	0 60	1 03	2 10	2 00	2 00
Leafy vegetables	0 17	0 06	0 09	0 06	0 11	0 03
Non leafy „	0 60	0 70	1 38	1 30	1 90	2 70
Fruits						
Ghee			0 08	0 16	0 20	0 40
Vegetable oil	0 10	0 10	0 27	0 40	0 40	0 60
Milk and milk products	0 34	2 40	1 90	2 80	3 90	4 10
Meat, fish and eggs	0 30	0 50	0 20	0 20	0 10	0 60
Sugar			0 17	0 20	0 25	0 60
Condiments	1 20	1 40	1 80	2 50	1 30	1 30

The main cereals consumed by all six groups were home-pounded rice, jawar and maize. The first three groups consumed ragi to some extent. Groups IV, V and VI took some wheat in addition to rice and jawar. Though most of the rice eaten was home-pounded, the fashion of taking milled rice is creeping in.

Pulses and cereals were the chief source of protein in the diet, milk, meat, fish and eggs being taken very sparingly. Fats were derived mostly from vegetable oils. All the six groups used vegetable oil for cooking. The first three groups do not take any ghee at all. The last three groups consumed some ghee, but its consumption was insignificant.

The intake of leafy and non-leafy vegetables was low and was not influenced by economic status. Fruits were absent from the dietary.

Ninety-two families did not take milk. In the higher-income groups some milk was taken with tea.

Table III shows calorie intake and the proportion of total calories supplied by protein, fat and carbohydrate —

TABLE III  
*Calorie intake and distribution*

Group	Total calories	Proteins	Fats	Carbo-hydrate	Cereals	Milk and milk products
I	2,640	10.6	3.4	82.2	90.0	0.2
II	2,680	11.2	4.2	82.7	94.0	0.9
III	2,630	11.3	6.8	83.2	87.9	1.6
IV	3,130	11.4	8.5	83.2	83.7	2.5
V	3,200	11.1	7.6	83.7	83.5	3.3
VI	3,050	11.6	8.2	84.3	85.3	3.6

Table IV shows that protein intake rose in all six groups with increase in income but the percentage of calories obtained from protein was more or less the same in all groups.

TABLE IV

*Average daily intake of nutrients per consumption unit (grammes)*

Group	PROTEIN		FATS		Carbohydrate	Calories	Calcium	MINERALS			VITAMINS		
	Total	Animal	Total	Animal				Phosphorus	Ca P ratio	Iron, mg	A, I U	B <sub>12</sub> , I U	C, mg
											Average intake for all groups		
I	68.0	2.1	9.0	0.3	529.0	2,640	0.20	1.64	1.82				
II	72.0	3.2	12.0	0.8	572.0	2,670	0.22	1.99	1.90				
III	72.0	2.2	19.0	2.8	532.0	2,620	0.23	1.82	1.65				
IV	87.0	3.6	28.0	7.3	632.0	3,130	0.50	1.92	1.38				
V	86.0	2.7	26.0	9.6	652.0	3,190	0.39	2.00	1.51				
VI	86.0	6.4	27.0	6.5	626.0	3,050	0.41	2.24	1.54			.	

The percentage of calories obtained from fat varied with income, the highest percentage being in the highest income group and lowest in group I. The same was true of carbohydrates. The percentage of calories derived from cereals was high in all groups and in general varied inversely with income.

Intake of protein is shown in Table IV. Total protein intake was satisfactory, but the amount of animal protein included in the diet was small. Most of the protein came from vegetable sources.

The consumption of total and animal protein was higher in the superior-income groups. Fat intake also varied with income. With a rise of income there was an increase in fat consumption, but this was low even in the higher-income groups. The improvement in diet and in the intake of various constituents was not regular after the level attained by group IV is reached.

Calcium intake was low in all the six groups and the calcium-phosphorus ratio was unsatisfactory.

Iron intake in all the groups ranged between 20 mg and 26 mg per head per day, as calculated on the basis of analyses given in Health Bulletin No 23.

However, the iron content of various foodstuffs differs according to the nature of soil and climatic conditions, and the amount of physiologically available iron in various foods is not exactly known. Hence it is difficult to say how far the diets were adequate as regards iron content. It is said that 'if a diet contains proteins, calcium, phosphorus and various vitamins in reasonably adequate amounts, its iron content will probably be adequate'. The diet of all the groups was deficient in vitamins and calcium.

The diets of all the groups were deficient in vitamin A and carotene, their values hardly reaching 520 I U. The B vitamins, calculated from Health Bulletin No. 23, gave the average figure of 490 I U per head per day. Most of vitamin B was obtained from rice and little from pulses. But the practice of washing rice repeatedly and throwing away the 'kanji' must diminish the vitamin values. Average figure for vitamin C consumption came to 25 mg per head per day. But, with regard to vitamin C, calculated figures often mean very little, since vitamin C value of foods as consumed is strongly influenced by methods of preparation.

#### FOOD DEFICIENCY DISEASES

Five hundred and twenty-seven persons out of 784 members of 149 families were examined for signs of deficiency diseases. Xerosis of the conjunctiva was present in all the income groups and was most prevalent in groups II, III and V —

TABLE V

#### *Percentage incidence of deficiency diseases*

Group	Number of subjects examined	Xerosis	Bitot's spots	Angular stomatitis	Glossitis
I	60	4.3		1.4	2.8
II	142	8.4		0.7	7.0
III	210	6.1	1.4	1.9	4.7
IV	53	3.7		3.7	
V	34	8.8			2.9
VI	19				.

Cases of angular stomatitis and glossitis were detected but not in large numbers

One thousand five hundred and seventy five boys from village schools in the area concerned were investigated for deficiency diseases. The results are shown in Table VI —

TABLE VI

*Percentage incidence of deficiency diseases in school children*

Number of subjects examined	Xerosis	Bitot's spots	Angular stomatitis	Glossitis
1,575	71	16	17	06

Here also the percentage incidence of xerosis was high, while that of Bitot's spots and angular stomatitis was relatively less

'Mottled enamel' of the type associated with fluorosis was observed in 43 boys. Eight of these came from one village (Achampet) in which there were only two wells for drinking purposes. The water from these wells was found to contain 15 parts per million of fluorine. The extent of fluorosis in Hyderabad State is a problem for further investigation

#### SUMMARY

1 A diet survey has been carried out in the Nizamabad district of Hyderabad. The survey included 147 families. The families were divided into six income groups

2 Intake of calories was satisfactory in all groups, but the diets were generally deficient as regards their contents of animal protein, fat, calcium and vitamins A and C. The quality of diet improved with increasing income

3 The incidence of xerophthalmia, evidence of vitamin A deficiency, was high

#### ACKNOWLEDGMENTS

We are grateful to Dr H Hyder Ali Khan, Director, Medical and Public Health Department, for his permission to publish this report and are greatly indebted to Dr M Farooq, Deputy Director of Public Health, for his help and guidance. We must thank Dr Aykroyd for revising the manuscript

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# INFLUENCE OF THE ANTERIOR LOBE OF THE PITUITARY GLAND ON CALCIUM METABOLISM \*

## Part I.

### CALCIUM BALANCE

BY

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[Received for publication, May 30, 1942.]

THE observed effects of hypophysectomy and administration of anterior lobe extracts on growth, and the marked skeletal changes in acromegaly and gigantism, indicate a relationship between anterior pituitary and calcium metabolism. Previous work on the subject has consisted mostly of the investigation of changes in blood-calcium level, the results of which have been conflicting and sometimes contradictory. Mere blood-calcium estimations give no information in regard to whether calcium is passing out into the excretory channels or into the bones (Bauer, Albright and Aub, 1929). Balance studies may be expected to yield more valuable and reliable information regarding any disturbance in the metabolism of any substance than analysis of its concentration in blood. Previous work on the influence of anterior pituitary on calcium balance has been, however, meagre and indecisive. The earliest work was reported by Medigreceanu and Kristeller (1911) who found that calcium was retained in acromegalics, but that immediately following treatment with anterior pituitary extract there was a loss of calcium with a rise in general metabolism. Pugsley and Anderson (1934) state that hypophysectomized rats on a low calcium diet showed a negative calcium balance which became positive on the administration of growth hormone, this change occurring with the resumption of growth. Struck and Szurek (1937), who noted increase in weight of a dwarf under treatment with growth hormone, also studied calcium

\* From a thesis presented to the University of Madras which formed the basis of the award of the degree of Master of Science

balance in the same patient and found that it was unchanged Teel and Cushing (1930), working on dogs, reported considerable increase in calcium excretion in the urine during the first few days following injection of growth hormone to normal animals Actual determination of calcium balance does not appear to have been made Johnston and Maroney (1939) investigated nitrogen as well as calcium balances in a group of children, among whom were six dwarfs and some normal children, who were given short-period treatments with 'Antutrin Growth' In some cases they observed increased retention of calcium, in others no effect and in still others decreased retention

In the present work calcium balance experiments were carried out on 32 albino rats and 11 guinea-pigs, the preparation employed being 'Antutrin Growth' made by Parke, Davis and Co The duration of the experiments varied from 7 to 11 days, excepting in one case in which it was 18 days The dosage employed for albino rats ranged from 0.2 c.c. to 0.5 c.c. and for guinea-pigs from 0.5 c.c. to 1 c.c. (1 c.c. = 10 units), administered daily intraperitoneally, in progressively increasing amounts within the limits mentioned The animals were especially bred for the purpose of this work so that suitable litter-mate pairs of known age, brought up under good dietetic and hygienic conditions, were available when required The food given to stock animals consisted of fresh cow milk, Bengal gram soaked overnight in water and green leaves of cauliflower and cabbage During the experimental period the diet consisted of fresh cow milk and Bengal gram (previously washed, dried and stored) soaked overnight in distilled water For the albino rats another diet, richer in calcium, was also used in a few experiments, referred to hereafter as 'special diet' This is a modification of Dyer's diet (Burn, 1937) and was made up as follows —

	Grammes
Bengal gram, powdered	45
Whole wheat, "	40
Dried milk (Klm)	20
Casein	9
Dried yeast	5
Calcium carbonate	1
Sodium chloride	0.5

These ingredients are very thoroughly mixed together and ground down into a paste with pulvis tragacanth 0.15 g. and warm 'ghee' 15 c.c., with the help of a little warm distilled water The resultant mass is non-sticky and can be rolled readily into balls of convenient size as required It can be stored in the refrigerator where it keeps well for weeks The rats eat this diet very eagerly The guinea-pigs, however, would not touch it even if the alternative were starvation

#### METABOLISM CAGES

These were of the usual pattern having a simple device for collecting urine and faeces separately and each having two food containers with 2-inch flanges By the use of these containers with somewhat high flanges scattering of food was reduced

to a minimum and at the same time the animals had no difficulty in eating or drinking out of them. An additional food container was also provided in the case of rat experiments for the rat to sleep in, in the absence of which the rat is likely to lie down in one of the other food containers and vitiate the experiment by passing urine and faeces in them.

#### EXPERIMENTAL PROCEDURE

A pair of litter-mate animals of the same sex was selected, one to receive the hormone and the other as control, except in four rat experiments where the same animal was studied during two consecutive periods, first control and then experimental. The animals were placed in their respective metabolism cages for a preliminary period varying from 2 to 5 days so that they might become accustomed to the strange conditions. The animals were then weighed and measured amounts of the food were placed in the containers. Milk was given in two instalments daily, each time just as much as the animal would drink completely in a couple of hours.

On the first day intraperitoneal injection of 'Antuitrin Growth' was given to the experimental animal, and the same quantity of re-distilled water to the control. Next morning the quantity of food left behind in each of the two cages was determined, the faeces and urine collected, food containers cleaned, fresh food put in and injections given. The procedure was repeated daily till the end of the experiment. The daily collection of faeces was dried in a hot-air-bath and stored. The urine collected was added to the washings of the cage obtained by spraying with acidulated distilled water (1 in 400 HCl) making a total volume of about 100 c.c. to 150 c.c. daily. To this 5 c.c. of 1 in 4 HCl were added to dissolve the precipitated salts. The fluid was then filtered and a further 5 c.c. of 1 in 4 HCl added, as the acid preserves the urine. The procedure was repeated daily and the fluid stored in a 2-litre bottle.

The food account of the last day of the experiment was taken in the evening and not next morning as on other days, the animal being starved during the interval, only distilled water being allowed, so that there was no food left behind in the alimentary tract at the end of the experiment next morning, when the faeces and urine were collected as usual and the animals weighed.

#### CALCIUM ESTIMATION

The calcium content of the collected urine and faeces and the foods used was estimated using standard methods of analysis. In the case of the Bengal gram an estimation was done for each lot bought from the bazaar. In the guinea-pig experiments analysis was done on the gram entire with husk, as these animals eat the gram as well as the husk. In the rat experiments the gram alone was analysed as rats do not eat the husk. With regard to milk, only one analysis was carried out for each balance experiment as, throughout one experimental period, the milk

used was invariably from the same cow milked under supervision and in whose food there was no material change during the period

## RESULTS OF INVESTIGATION

*Albino rats*—Table I gives the figures for total calcium intake and calcium retention in 12 experiments in which litter-mates of the same sex were used as controls

TABLE I

*Calcium intake and retention, litter-mate being used as control*

Litter of rats	Identification number	Sex	C=control. E=experimental	Age in days *	Duration of experiment in days	O D = ordinary diet S D = special diet	Total calcium intake in mg	CALCIUM RETENTION IN MG		Excess retention per cent by the treated rat †
								Total	Per 100 mg of calcium intake	
IJ	41	M	C	62	9	O D	210	107	51	33
IJ	40	M	E	62		O D	214	145	68	
IJ	46	F	C	63	11	O D	243	131	54	29
IJ	44	F	E	63		O D	245	172	70	
L	53	F	C	75	9	O D	246	122	50	22
L	54	F	E	75		O D	240	152	61	
N	61	F	C	76	8	S D	250	52	20	100
N	60	F	E	76		S D	325	131	40	
H	36	M	C	80	18	O D	558	277	50	24
H	35	M	E	80		O D	558	346	62	
C	21	M	C	89	10	O D	309	157	51	45
C	23	M	E	89		O D	270	201	74	

\* Mean of the ages at the beginning and end of the experiment

† The excess calcium retained by the treated animal expressed as a percentage of the amount retained by the control.

TABLE I—concl'd

Litter of rats	Identification number	Sex	C=control E=experimental	Age in days *	Duration of experi- ment in days <sup>†</sup>	O D = ordinary diet S D = special diet	Total calcium intake in mg	CALCIUM RETEN- TION IN MG		Excess retention per cent by the treated rat †
								Total	Per 100 mg of calcium intake	
N	62	F	C	91	8	S D	367	114	31	42
N	63	F	E	91		S D	380	166	44	
C	24	F	C	111	7	O D	186	35	18	111
C	27	F	E	111		O D	203	78	38	
L	50	M	C	155	8	S D	448	94	21	62
L	51	M	E	155		S D	463	157	34	
N	65	M	C	198	10	S D	538	170	32	
N	64	M	E	198		S D	567	175	31	
O	71	M	C	204	11	S D	454	64	14	107
O	70	M	E	204		S D	346	100	29	
O	73	F	C	218	10	O D	307	14	5	580
O	72	F	E	218		O D	306	105	34	

\* Mean of the ages at the beginning and end of the experiment

† The excess calcium retained by the treated animal expressed as a percentage of the amount retained by the control.

In 11 out of the 12 experiments the animals treated with 'Antutrin Growth' retained more calcium than the controls. With regard to the experiment (rats 64 and 65) in which such a result was not obtained, it may be mentioned that the daily calcium intake of both these animals was higher than that of any other pair and that the control 65 retained far more calcium than the controls 71 and 73 of about the same age, used in other experiments.

In three experiments (rats 2, 3 and 6) the same rat was studied during two periods, first the control period followed immediately by the experimental period

In every case there was increased calcium retention during the experimental period (*vide* Table II) In one experiment (rat 7) both were control periods, no injections whatever being given in the first period and only injections of re-distilled water in the succeeding period. The values of calcium retention remained practically the same during both periods

TABLE II.

*Calcium intake and retention, the same rat being studied during two periods*

Litter of rats	Identification number	Sex	C = control period. E = experimental period	Mean age in days *	Duration of experiment in days	O D = ordinary diet S D = special diet	Total calcium intake in mg	CALCIUM RETENTION IN MG		Excess retention per cent during the experi- mental period †
								Total	Per 100 mg of calcium in- take	
A	2	M	C	85	7	O D	190	69	36	47
A	2	M	E	94	11	O D	346	183	53	
A	3	M	C	85	7	O D	194	38	20	60
A	3	M	E	94	11	O D	344	111	32	
A	6	F	C	85	7	O D	196	64	33	36
A	6	F	E	94	11	O D	342	154	45	
A	7	F	C	85	7	O D	190	68	36	}
A	7	F	C	94	11	O D	346	127	36	

\* and †—See under Table I

Rats 2, 3 6 and 7 were litter-mates experimented on simultaneously. The result obtained in the case of rat 7 clearly shows that the increased calcium retention by rats 2, 3 and 6 after treatment with 'Antuitrin Growth' was due to the hormone and not any other factor.

Table III gives the average values and the ranges of calcium retention, obtained in 15 out of 16 experiments, the experiment on rat 7 being omitted as here both the periods were control periods.

TABLE III

*Average values and ranges of calcium retention*

	RETENTION IN MG PER 100 MG OF CALCIUM INTAKE		RETENTION IN MG PER 100 g WEIGHT OF ANIMAL PER DAY	
	Average	Range	Average	Range
Control rats	32	5 to 54	7.0	0.9 to 12.2
Experimental rats	48	29 to 74	10.4	5.0 to 14.7

The average value of calcium retention by the rat treated with 'Antutrin Growth' is 50 per cent above that of the control. The range of values, however, is very wide both in the control and in the experimental animals. That this does not take away the significance of these averages is clear from an examination of Tables I and II. In the first place the variations are due to a considerable extent, to the age differences of the animals and, secondly, in every experiment with but one exception, the rat treated with 'Antutrin Growth' showed a higher positive calcium balance than its control.

The mean ages of the rats varied from 62 to 218 days and so included animals in the most rapid stage of growth, a less rapid stage and the 'plateau' stage which is reached at about the age of 150 days. It will be seen from Tables I and II that there is a general tendency for calcium retention to fall with increasing age in both the control and the treated animals. If the excess retention by the treated animal, per 100 mg of calcium intake, is expressed as a percentage of the retention by the control, it tends to be generally higher the older the animal, suggesting that in the older animal the stimulus offered by the injected 'Antutrin Growth' generally tends to produce a greater retention relatively to that of the control, than in the case of younger animals.

The duration of injection varied from 7 to 11 days except in one experiment in which it was 18 days. The differences in duration within these ranges did not influence calcium retention to any appreciable extent. This is evident when the results of the experiments on rats 36 and 35 with a duration of 18 days, are compared with those on 53 and 54 with a duration of 9 days, the ages of both pairs being practically the same. The value for calcium retention per 100 mg of calcium intake and excess retention percentage do not show any marked variations.

The sex of the animal does not appear to have affected calcium retention in any notable manner.

The daily calcium intake of the rats varied from 22 mg to 31 mg in the case of those whose food consisted of Bengal gram and milk, and from 31 mg to 58 mg in those which were given the special diet. These variations did not exert any notable effect on the percentage of calcium retained.



A reduction in faecal calcium in the rat treated with 'Antutrin Growth' was a consistent feature in all cases where the calcium retention of the treated rat was higher than that of the control. It will be seen from Table IV that in 14 experiments on rats, out of a total of 15, faecal calcium in the treated rat was lower than in the control. In 10 of these, the reduction was considerable, the increased retention being largely attributable to this factor. That this low faecal calcium plays an important part in raising calcium retention is indicated by the results of experiments on rats 21 and 23 and 71 and 70 (*vide* Tables I and IV). In both these experiments, in spite of a considerably lower calcium intake, the treated animal retained much more calcium than the control, the faecal calcium being very much lower.

TABLE IV

*Average daily calcium intake and excretion*

Identification number of rat	C=control E=experi- mental	Calcium intake in mg per day, calculated	CALCIUM EXCRETION IN MG PER DAY, CALCULATED		
			Faeces	Urine	Total
41	O	23	8.9	2.6	11.5
40	E	24	4.5	3.1	7.6
46	C	22	8.7	1.5	10.2
44	E	22	4.7	1.9	6.6
53	C	27	11.7	2.1	13.8
54	E	28	8.1	2.7	10.8
36	C	31	13.3	2.3	15.6
35	E	31	9.0	2.8	11.8
61	C	31	22.0	2.8	24.8
60	E	40	21.1	3.1	24.2
21	C	31	12.8	2.4	15.2
23	E	27	4.5	2.4	6.9
62	C	46	29.5	2.1	31.6
63	E	48	23.5	3.3	26.8
24	C	27	18.3	3.6	21.9
27	E	29	14.3	3.6	17.9
50	C	56	42.0	2.3	44.3
51	E	58	35.5	2.8	38.3
65	C	54	34.1	2.7	36.8
64	E	57	36.6	2.6	39.2
71	C	41	32.6	2.8	35.4
70	E	31	19.7	2.6	22.3
73	C	31	25.4	3.9	29.3
72	E	31	17.6	2.5	20.1
2	C	27	13.6	3.7	17.3
2	E	31	11.6	3.3	14.9
3	C	28	19.9	2.4	22.3
3	E	31	18.1	3.1	21.2
6	C	28	16.0	2.9	18.9
6	E	31	14.5	2.6	17.1

Although a larger calcium intake is not the chief factor responsible for the increased calcium retention, it is a fact that in most of the experiments the rat treated with 'Antutrin Growth' had a higher calcium intake than the control. Where the treated rat was heavier than the control, an increased calcium intake is readily explained by the greater food intake of a sturdier animal. There are, however, six cases in the series (*vide* Table V) in which the treated animal was less heavy than the control, in none of which was the calcium intake of the treated animal appreciably less than that of the control. On the other hand, in four of these cases the calcium intake was higher.

TABLE V

*Calcium intake in six experiments where the control rat was heavier*

Identification number of rat	C=control E=experimental	WEIGHT IN G		Total calcium intake in mg
		Beginning of experiment	End of experiment	
53	C	123	137	246
54	E	118	136	249
36	C	159	188	558
35	E	155	188	558
62	C	145	156	367
63	E	142	155	380
50	C	192	199	448
51	E	188	198	463
65	C	201	224	538
64	E	194	226	567
73	C	143	149	307
72	E	131	145	306

The results of balance experiments on rats 73 and 72 have a special interest in view of the very high excess retention percentage of the treated animal, namely 580 (*vide* Table I). The rats of litter 'O', of which 71 and 70, 73 and 72 have been used in this work, were small animals as can be seen on a comparison with rats 65 and 64 of litter 'N' (*vide* Table VI). Rat 72 was particularly small. Injections of 'Antutrin Growth' appear to have stimulated the dormant growth of rat 72 to an extraordinary degree as indicated by the very large value obtained for the excess retention percentage, the previous retarded growth being presumably due to a deficiency of the 'Growth' hormone. This result is in conformity with the observation of Johnston and Maroney (*loc cit*) that in the field of hormones 'an effect will be conditioned by the presence or absence of a defect'. They observed that children who are pituitary dwarfs show increased retention of nitrogen and calcium on administration of 'Growth' hormone.

TABLE VI

Litter	Number	Sex	C=control E=experimental	Age in days	Weight in g
N	65	M	C	203	224
N	64	M	E	203	226
O	71	M	C	209	189
O	70	M	E	209	185
O	73	F	C	213	143
O	72	F	E	213	131

## GUINEA-PIGS

Six experiments were carried out on guinea-pigs in which 11 animals were used, 6 experimental and 5 control. The animals were all young being between 57 and 127 days old. The duration of experiments varied from 8 to 11 days. In 4 experiments 'Antutrin Growth' injections caused increased calcium retention. The excess retention percentage varied from 23 to 30 with an average of 28. For a similar age group in albino rats comprising 11 experiments the values for excess retention percentage varied from 22 to 111 with an average of 50. No conclusions can, however, be drawn regarding the relative responses of these kinds of animals as the number of guinea-pig experiments has been small.

In 2 out of 6 experiments, 'Antuitrin Growth' injections did not produce increased calcium retention. In both, the treated animal had definitely lost weight during the period of investigation, whereas in all other cases the weight had increased. These two experiments suggest that a normal state of nutrition is essential for 'Antuitrin Growth' injections to cause increased calcium retention. The loss of weight was apparently due to the solitary life in the metabolism cages, the animals differing in their reactions to this new environment. Guinea-pigs usually take a longer time to adjust themselves to the strange mode of life in the cages than albino rats. Some of them never attain their normal food intake, many such animals having been rejected after trial. In one of the 4 experiments which gave positive results 3 litter-mate guinea-pigs were used, two males and one female, one male being the control and the other two experimental animals. The results (*vide* Table VII) indicate that male and female guinea-pigs, under identical conditions, do not exhibit any differences in their response to 'Antuitrin Growth' injections.

TABLE VII

*Calcium intake and retention in 3 litter-mate guinea-pigs*

Litter	Identification number	Sex	C=control E=experimental	Mean age in days	Total calcium intake in mg	CALCIUM RETENTION IN MG		
						Total	Per 100 mg intake	Excess retention, per cent
VI	18	M	C	123	705	308	44	
VI	17	M	E	123	727	404	56	27
VI	19	F	E	123	729	416	57	30

*Excretion of calcium by albino rats and guinea-pigs*—In all the experiments on albino rats, with one exception (rats 65 and 64), in which the treated animal did not show increased calcium retention, the amount of faecal calcium of the treated rat was less than that of the control (Table IV). The urinary calcium, on the other hand, did not show any constant or marked change. In most cases it was slightly increased in the treated animals, while in other cases there was either no change or even a decrease. The reduction of calcium in the faeces accounts for the increased calcium retention by the treated animals. As the important channel of calcium excretion is the large bowel, it is, of course, not possible to say whether the reduction in faecal calcium is brought about by increased absorption from, or diminished excretion into, the bowel.

Guinea-pigs differ from albino rats in the relative amounts of calcium present normally in faeces and urine. In the control animals the ratio between faecal and urinary calcium was approximately 2 to 3 in the case of guinea-pigs and 8 to 1 in the case of rats (*vide* Table VIII). Thus, although in the case of guinea-pigs a very much smaller fraction of the total calcium is normally found in the faeces, the increased calcium retention is still accounted for, to a large extent, by diminished faecal calcium.

TABLE VIII

*Daily faecal and urinary calcium in rats and guinea-pigs*

	RATS		GUINEA-PIGS	
	Control (17 animals)	Treated (15 animals)	Control (5 animals)	Treated (6 animals)
Daily average faecal calcium of an animal in mg	20.1	16.2	18.0	13.2
Daily average urinary calcium of an animal in mg	2.6	2.8	27.2	25.4

#### SUMMARY AND CONCLUSIONS

1 The effects of the growth hormone of the anterior pituitary on calcium metabolism were investigated. The preparation used was 'Antutrin Growth' made by Parke, Davis & Co.

2 Calcium-balance experiments, using litter-mate controls of the same sex, were carried out on 32 albino rats (15 experimental and 17 control) and 11 guinea-pigs (6 experimental and 5 control) for periods ranging from 7 to 18 days. Daily intraperitoneal injections of 'Antutrin Growth' were given to experimental animals and re-distilled water to controls. The calcium content of the food, faeces and urine was estimated in all cases.

3 In all the experimental rats, with the exception of one, the calcium retention in the treated animal was considerably more than that in the control, the average value being 50 per cent above. In 4 out of 6 experiments on guinea-pigs the average value was 27 per cent above that of the control. The two guinea-pigs that did not give such a response lost weight in the course of the experiment.

4 There appears to be a general tendency for calcium retention to diminish with age in both treated and control rats. On the other hand, the excess retention percentage (the excess calcium retained by the treated animal expressed as a percentage of the amount retained by the control) tends to increase with age, within the age limits (57 and 218 days) of the albino rats used in this work.

5 The faecal calcium was significantly low in the treated animals as compared with the respective controls. The urinary calcium did not show any marked or constant differences.

6 The duration of injections within the limits of 7 and 18 days, sex differences, and the variations in the daily calcium intake do not appear to have influenced calcium retention to any appreciable extent.

#### ACKNOWLEDGMENTS

It is a pleasure to record my thanks to Professor B. T. Krishnan, Head of the Department of Physiology, Madras Medical College, for suggesting this investigation and for his guidance and help in the conduct of this work, and also to Professor K. Venkatachalam Pillai, Research Officer in Pharmacology, and to Mr. A. N. Ratnagiriswaran, Research Chemist, for placing the facilities of their laboratory at my disposal and for their interest and encouragement throughout this work. I also desire to express my thanks to Messrs. Parke, Davis & Co. for their prompt deliveries of the hormone 'Antuitrin Growth' when required and for supplying voluntarily, free of cost, several tubes of this expensive hormone in the initial phases of this work.

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## ON SERUM PHOSPHATASE

### Part I

#### THE SERUM PHOSPHATASE IN PULMONARY TUBERCULOSIS AND THE EFFECT OF INGESTION OF VITAMIN C UPON IT

BY

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[Received for publication May 20, 1942 ]

SINCE Robison (1923) observed the hydrolysis of calcium and barium hexosemonophosphates by emulsin, the theory of the mechanism of calcification has had a different orientation. In the new scheme, mainly developed by the Robison school, phosphatases play an important rôle. If we consider the phosphatase distribution in adult tissues and body fluids, we find that blood has a very low phosphatase value (Kay 1932). The phosphatase in erythrocytes appears to be different from the phosphatase of plasma or serum. In any disease in which there is lesion, with consequent breakdown of any tissue with higher phosphatase, the plasma or serum phosphatase value is increased. Kay (1930) found that in a large number of bone, thyroid and renal diseases, the plasma phosphatase is considerably increased. High plasma or serum phosphatase is usually found in rickets, hyperthyroidism and Paget's disease (Kay, 1932, Bodansky and Jaffe, 1934). In rickets serum-phosphatase estimation has been reported to be a better criterion of the severity of the disease and the course of healing than the estimation of serum calcium or phosphorus (Bodansky and Jaffe, 1934a, Andersen, 1935). Roberts (1923) first observed an increase in the serum-phosphatase value in obstructive jaundice. This has since been confirmed by Armstrong, King and Harris (1934),



Bodansky and Jaffe (1934b), Green, Shattuck and Kuplowitz (1934) High serum-phosphatase activity is usually found in diseases where there is considerable disorder in calcification (Albright, Aub and Bauer, 1934, Gutman, Swenson and Parsons, 1934) Blood-phosphatase activity is raised in osteoblastic type of osteogenic sarcoma (Frauseen and Mclean, 1935) Morris and Peden (1937) have determined the serum-phosphatase value in a number of bone diseases They are of opinion that by itself the determination of phosphatase is of little diagnostic or prognostic value They opine, however, that in conjunction with clinical, radiological and biochemical findings it may yield considerable assistance in diagnosis Repeated determinations may give valuable ideas about the efficiency of treatment in some bone diseases Gutman and Gutman (1938) found increased 'acid' serum phosphatase in metastasizing carcinoma of the prostate Albers (1938) also found increased serum phosphatase in cancer with metastasis Muller (1938) obtained similar results He also found increased 'alkaline' phosphatase in Paget's disease De and Cayal (1939) reported increased blood phosphatase in pregnancy Iwatsuru and Nanja (1939) found increased serum phosphatase in myeloid leukemia

It is expected that in pulmonary tuberculosis with parenchymatous lesions where there is serious disturbance in calcium metabolism, the serum phosphatase should be increased

In the course of an investigation on the therapeutic value of vitamin C in pulmonary tuberculosis, it was thought desirable to find out the phosphatase activity of serum in this disease and to compare it with normal values Koldajew and Altschuber (1933) found a small increase in 'blood' phosphatase in tuberculosis in man It was also the object of the investigation to inquire into the effect of the administration of comparatively large doses of vitamin C, on the serum-phosphatase activity in pulmonary tuberculosis

#### EXPERIMENTAL

We have followed Bodansky's (1932, 1933, 1937) method for determination of the serum phosphatase Patients with pulmonary tuberculosis in the general and isolation wards of the Patna Medical College Hospital were selected for the phosphatase study Normal persons of about equal social status were taken as controls Venous blood was drawn with a sterile dry syringe and quickly transferred to a sterile tube and set for separation of the serum After the serum had separated, it was transferred to a sterile centrifuge tube and centrifuged at 3,000 revolutions per minute for 15 minutes The clear serum free from blood corpuscles was then carefully pipetted into another clear sterile tube and the phosphatase activity estimated The time taken between the drawing of blood and incubation of the serum with the substrate was in no case more than one hour and in all the determinations about an equal time was taken

The patients were divided into two groups One group received no additional vitamin C and served as controls The other group received orally 200 mg to 250 mg vitamin C daily in the form of 10 g of sun-dried pulp of Indian gooseberry (*Phyllanthus emblica*) The oral administration of the additional vitamin C was

continued for ten consecutive days. The same diet and medical treatment were given to both groups. After this ten day period the serum-phosphatase activities of the controls and of those receiving additional vitamin C were again determined. All the cases were males except one in the pulmonary tuberculosis group who was a female.

## RESULTS

From Table I it will be observed that normal serum-phosphatase activity is lower than the serum-phosphatase activity in pulmonary tuberculosis.

TABLE I

*Serum phosphatase in normal males and in male patients suffering from pulmonary tuberculosis*

Serial number	NORMAL		PULMONARY TUBERCULOSIS	
	Age	Phosphatase units	Age	Phosphatase units
1	22	2.28	24	1.56
2	24	1.00	30	2.36
3	30	2.44	20	4.96
4	35	1.08	20	1.72
5	35	0.68	20	1.56
6	20	2.16	*25	2.44
7	27	0.80	35	3.16
8	35	2.64	30	7.64
9			26	2.36
10			40	5.44
11			18	4.68
12			20	9.96
13			32	7.88
14			35	6.44
15			35	4.80
16			30	3.08
17			25	5.28
18			25	3.84
19			24	1.32
20			22	3.84
21			18	2.88
22			20	2.56
23			19	1.60
Mean		1.63		3.96

\* Female

Both the average phosphatase activity and the range are higher in the case of pulmonary tuberculosis patients. Average normal serum-phosphatase activity is 1.63 units, the range of activity being 0.68 to 2.64 units. The corresponding figures for pulmonary tuberculosis patients are 3.96 units and 1.32 to 9.96 units.

*Statistical analysis of the above results*

Difference between the two means	= 2.33
Standard error of difference	= 0.54
$t$	= 4.31

The probability of obtaining such a value of  $t$  by chance alone is less than one in 370. Thus, it is very unlikely that the order of the magnitude of the serum-phosphatase values observed in normals and in pulmonary tuberculosis should occur as a matter of chance. Therefore, serum phosphatase is significantly higher in pulmonary tuberculosis than in normals.

Oral administration of 200 mg. to 250 mg. of vitamin C in the form of *Phyllanthus emblica* powder had a decided action in lowering the serum-phosphatase activity of pulmonary tuberculosis patients as compared to the serum-phosphatase activity of controls receiving no additional vitamin C (Tables II and III) —

TABLE II

*Serum phosphatase in controls*

Number	Age	PHOSPHATASE UNITS	
		Before experiment	After 10 days
1	30	3.08	3.64
2	20	4.96	5.04
3	20	1.72	4.20
4	18	1.56	2.28
5	25	2.44	4.56
6	35	3.16	1.52
7	19	1.60	2.48
Mean		2.64	3.40

TABLE III

*Serum phosphatase in pulmonary tuberculosis patients  
on vitamin C supplement*

Number	Age	PHOSPHATASE UNITS	
		Before experiment	After 10 days
1	25	5.28	1.04
2	24	1.32	4.28
3	22	3.84	1.00
4	18	2.88	1.88
5	20	2.56	3.82
6	26	2.36	1.00
7	18	4.68	0.92
8	20	9.96	2.16
9	32	7.68	9.36
10	35	6.44	1.40
11	35	4.80	1.76
12	30	7.64	2.56
13	30	2.36	1.08
14	24	1.56	1.52
Mean		4.53	2.40

After this ten-day period the serum-phosphatase activity in the controls was lower in only one case (14.3 per cent) and increased in all the other cases (85.7 per cent). The average value rose from 2.64 units to 3.40 units. In pulmonary tuberculosis, on the other hand, the phosphatase activity was lower in ten cases (71.4 per cent) and increased only in three cases (21.4 per cent), while in one it was at the previous level. The average value fell from 4.53 units to 2.41 units. Thus, in pulmonary tuberculosis patients receiving from 200 mg to 250 mg additional vitamin C, there was an appreciable drop in the serum-phosphatase activity in most of the cases whilst the reverse was the case in the controls.

### Statistical analysis of Table III

Difference of the two means	= 2.13
Standard error of difference	= 0.89
$t$	= 2.40

The probability of obtaining such a value of  $t$  by chance alone is less than five in a hundred. Thus there is a significant fall in the serum-phosphatase value in pulmonary tuberculosis after the administration of 200 mg to 250 mg of vitamin C.

### DISCUSSION.

We can now say that serum-phosphatase activity of patients suffering from pulmonary tuberculosis is generally higher than that of normals although some of the values obtained approach the normal values. What is of greater significance is the fact that the comparatively high serum-phosphatase value in pulmonary tuberculosis is considerably lowered by oral administration of 200 mg to 250 mg vitamin C given as dried *Phyllanthus emblica* pulp. Apparently, this lowering of serum phosphatase is due to the vitamin C content of the powder. There is evidence to believe that this action of the vitamin C may be re-inforced by some other constituents of *Phyllanthus emblica*, possibly vitamin P. We do not consider that this lowered phosphatase activity is due to the inactivation of phosphatase by a vitamin C copper complex. Any such action would be counteracted by the presence in blood of glutathione and other sulphydryl compounds (cf. Giri, 1938). An increase in plasma ascorbic acid would neither activate nor retard serum phosphatase activity (cf. King and Delory, 1938, Thannhauser, Riechel and Grattan, 1938).

A large increase in phosphate ions would tend to inactivate serum phosphatase but no such increase of phosphate ions may be expected by the daily administration of 10 g of the powder. Similarly, an increase in calcium ions would lower the phosphatase activity but such a probability may also be discounted.

The lowered serum-phosphatase activity is due to the beneficial action of the large doses of vitamin C supplemented by the probable action of vitamin P\*, on the tuberculosis patients. Scoz, Cattaneo and Gobricelli (1937) have reported that in vitamin C deficient guinea-pigs, an increased intake of the vitamin results in a decrease of blood phosphatase with an increase in bone phosphatase. We may presume that a similar action takes place in the tuberculosis patients also. If the administration of large doses of vitamin C does increase the bone phosphatase this would mean a great boon to the tuberculosis patients. The bone phosphatase is an important link in calcification and increase of bone phosphatase in tuberculosis patients would result in smaller decalcification which would be a considerable gain to tuberculosis patients.

Not only would serum-phosphatase determinations be of help, along with other laboratory findings, in the diagnosis of tuberculosis but these determinations at regular intervals would certainly give, in conjunction with the usual laboratory tests a valuable idea of the prognosis of the disease.

#### SUMMARY

Serum phosphatase in pulmonary tuberculosis is generally higher than of normals.

Oral administration of large doses of natural vitamin C lowers the serum phosphatase of pulmonary tuberculosis patients.

#### ACKNOWLEDGMENT

We are indebted to Dr S M Ghosh, M B, B S, for the statistical analysis.

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\* We are in a position to state that the action of *Phyllanthus emblica* is due to the combined activity of vitamin C and vitamin P. The results of the investigations concerning this issue will be communicated later.

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## ON THE SUITABILITY OF THE GUINEA-PIG METHOD OF DIGITALIS ASSAY IN INDIA

BY

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[Received for publication, September 11, 1942 ]

SINCE the earliest days of biological standardization, the assay of the digitalis group of drugs has offered considerable difficulties. Various methods, such as 'frog method' of Houghton (1896), 'pigeon-emesis method' of Hanzlik (1929), 'dog method' originally described by Richaud (1914), 'cat method' of Hatcher and Brody (1910) and 'guinea-pig method' of Knaff-Lenz (1926) have been employed by various workers in different countries, but comparable results were seldom obtainable even under the best of laboratory conditions. In 1916, U S P IX first recognized officially the biological assay method for digitalis and accepted the 'one-hour frog method' as the standard method for comparison of all unknown preparations of digitalis. At the Edinburgh Conference held in 1924 under the auspices of the Health Committee of the League of Nations, the 'four-hour frog method' and the 'cat method' of Hatcher and Brody (*loc cit*), as modified by Professor Magnus of Utrecht, were recommended for adoption. The B P 1932 has recommended three methods for official adoption (1) 'twelve-hour-lethal-dose-frog method', (2) modified 'cat method' and (3) 'guinea-pig method' introduced by Professor Tiffeneau of Paris.

Chopra and De (1926) pointed out that it was difficult to get a suitable species of frog at all seasons of the year in India. The most easily available species in Calcutta is *Bufo melanostictus*, on which digitalis produces unreliable results. Besides, the seasonal variation in the sensitiveness of these frogs was found to be much more marked than what has generally been reported in the literature with such species of frogs as *Rana temporaria* or *Rana pipiens*. The 'frog method' was



therefore considered unsuitable for routine assay work at this Laboratory, although Bhatia and Lal (1934) more recently reported on the suitability of this method in India

The 'cat method' of the B P 1932 has been generally considered the method of choice in the Biochemical Standardization Laboratory and has recently been adopted for inclusion in the U S P XII (1942). The 'guinea-pig method' of assay, however, has seldom been used outside France. In view of the easy availability of guinea-pigs at all times of the year and in view of its cheapness compared to cats, which, as is well known, cannot be bred satisfactorily in captivity, it was considered worth while to investigate the possibilities of its adoption as a routine method of standardization in Indian laboratories. The present paper embodies the findings of this investigation.

#### EXPERIMENTAL DETAILS

Experiments were performed under standard conditions in order to eliminate all extraneous factors capable of vitiating the result. Guinea-pigs were anaesthetized with intraperitoneal urethane (25 per cent solution, 1.8 g/kg body-weight doses, three hours before the experiment started) and maintained at 38°C for the duration of the perfusion. Healthy male animals were used from the stock of the Central Research Institute, Kasauli, and their weight kept within the range of 600 g to 800 g. The diluted tincture was perfused through one of the jugular veins at a uniform rate of 0.5 c.c. per minute and the cardiac effect examined by palpation over the thoracic wall. Within 8 to 11 minutes of perfusion when the apex beat ceased to be palpable, a fine needle was introduced into the point of the heart and its movements further observed through oscillations of the needle. With the onset of fibrillation, the flow was reduced to 0.25 c.c. per minute and this was further reduced to 0.1 c.c. per minute when the heart became intermittent. The perfusion was continued till complete cessation of all cardiac pulsations. Neither artificial respiration nor blood pressure record was maintained. By this simple procedure, the different phases of digitalis action could be observed and the end-point accurately determined.

The standard tincture was prepared with dehydrated alcohol by continuous Soxhlet extraction for 6 hours from the International digitalis powder of 1936 containing 1 unit per 80 mg of powder. The stock tincture was adjusted to contain per cubic centimetre 1 unit of digitalis activity in 70 per cent alcohol\* (this being also the concentration of alcohol in commercial samples of tincture of digitalis), and stored at 0°C during the entire period of investigation. The tincture was further diluted to 1 in 8 with normal saline before use (representing thereby 0.125 unit per cubic centimetre) and perfused at body temperature. The whole series of tests were terminated within a week of the preparation of the tincture.

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\* The B P process for the preparation of the tincture from the International standard digitalis powder gives it an alcohol concentration of about 50 per cent only.

TABLE I

*Showing results of continuous intra jugular perfusion  
of standard digitalis tincture (1 in 8)  
on 30 guinea-pigs*

Guinea pig, male	Weight, g	Quantity perfused c.c.	Lethal dose per kg c.c.	Equivalent units
1	2	3	4	5
1	725	6.60	9.10	1.13
2	705	6.10	8.65	1.08
3	735	6.60	8.97	1.12
4	665	6.10	9.17	1.14
5	720	6.70	9.30	1.16
6	710	6.40	9.01	1.12
7	700	6.30	9.00	1.12
8	755	6.80	9.00	1.12
9	740	6.60	8.91	1.11
10	675	6.00	8.88	1.11
11	795	7.30	9.19	1.14
12	790	7.00	8.86	1.10
13	730	6.60	9.04	1.13
14	620	5.60	9.03	1.12
15	710	7.00	9.85	1.23
16	680	6.50	9.55	1.19
17	668	5.80	8.08	1.08
18	665	5.80	8.72	1.09
19	659	5.80	8.80	1.10
20	622	6.00	9.04	1.20
21	800	6.90	8.62	1.07
22	778	6.50	8.35	1.04
23	750	6.30	8.40	1.05
24	750	7.00	9.33	1.16
25	715	6.60	9.23	1.15
26	713	6.30	8.83	1.10
27	700	6.60	9.42	1.17
28	723	6.80	9.40	1.17
29	663	6.60	9.95	1.24
30	670	6.15	9.17	1.14
Mean lethal dose 9.07 c.c./kg			Mean unitage 1.129	
Standard deviation* = $\pm 0.381$			Standard error† = $\pm 0.069$	

\* Calculated from the formula,  $\sigma = \sqrt{\frac{\sum d^2}{n-1}}$

† Calculated from the formula,  $\epsilon = \sqrt{\frac{\sum d^2}{n(n-1)}}$

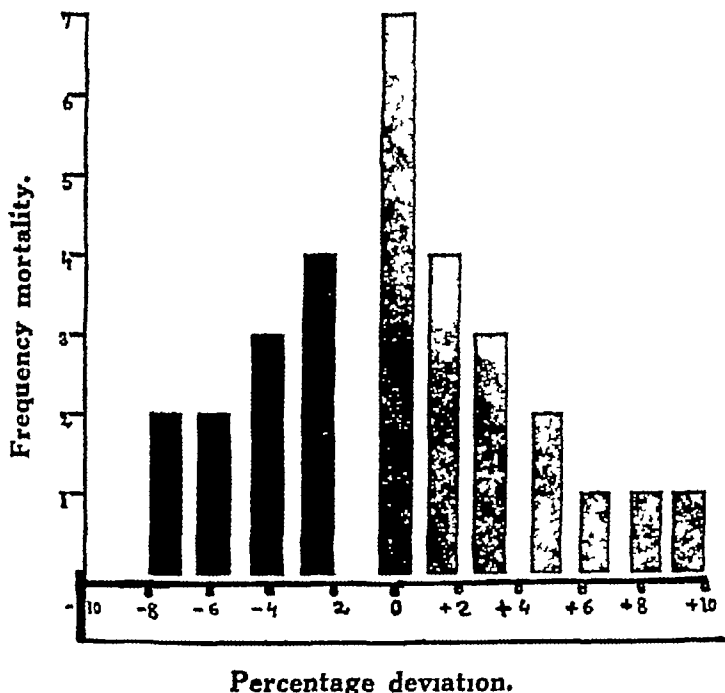
In Table II, the various lethal doses have been grouped into different ranges. The percentage frequency of mortality for each range of doses has also been worked out and integrated.

TABLE II.

*Showing the percentage frequency and integrated percentage frequency according to different lethal dose range*

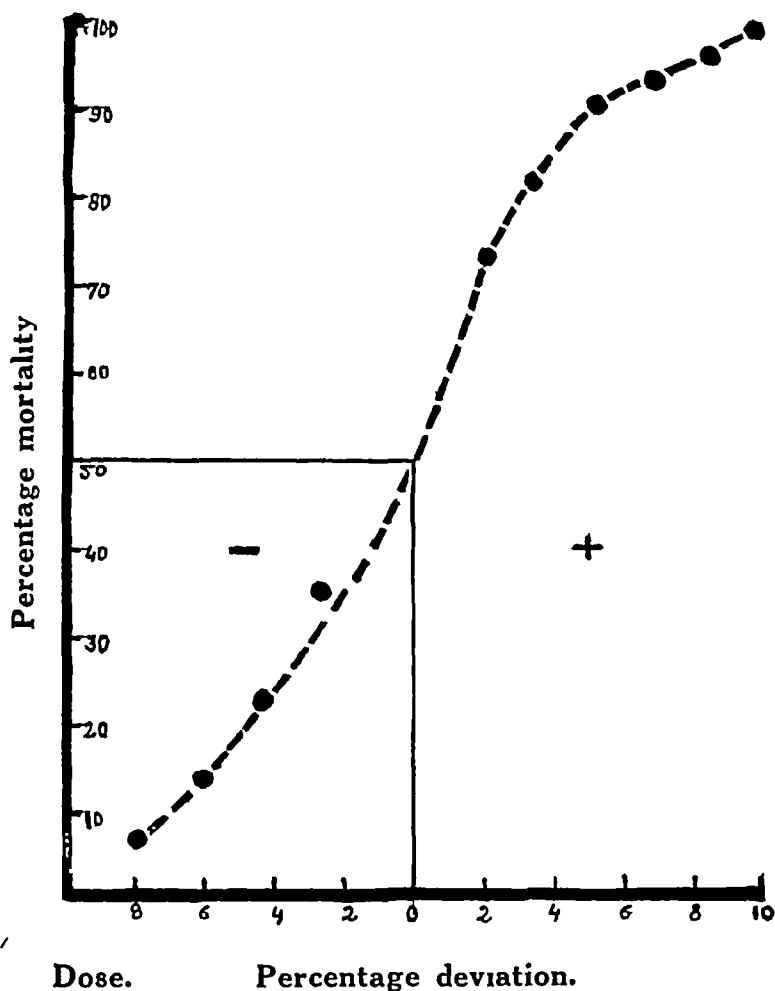
Lethal dose range in c c	Number of animal killed	Percentage mortality frequency	Percentage mortality frequency integrated
1	2	3	4
8.35—8.50	2	6.6	6.6
8.50—8.65	2	6.6	13.2
8.65—8.80	3	10.0	23.2
8.80—8.95	4	13.2	36.4
8.95—9.10	7	23.3	59.7
9.10—9.25	4	13.2	72.9
9.25—9.40	3	10.0	82.9
9.40—9.55	2	6.6	89.5
9.55—9.70	1	3.3	92.8
9.70—9.85	1	3.3	96.1
9.85—10.00	1	3.3	99.4

The results in the first two columns of Table II have been reproduced in Graph 1. The number of animals killed by each range of dose has been plotted as ordinates, and the lethal-doses range known by their standard deviation percentages as abscissæ. In spite of the relatively small number of animals used for this study, it will be observed that the chart resembles that of Behrens (1929) obtained with strophanthin on frogs.



GRAPH 1 Frequency distribution chart. Each pillar represents the effect of one range of doses, the distribution of which has been calculated from the percentages of the standard deviation from the mean lethal dose, 0.

In Graph 2, a dose-mortality curve has been constructed with the figures of the integrated mortality frequency as ordinates and the standard deviation percentages of the doses as abscissæ. The curve is steeper in the zone of the median lethal dose, and has a much lesser spread than similar curves obtained by Trevan (1927) with digitalis on frogs and by Behrens (*loc cit*) with strophanthin.



GRAPH 2 Dose mortality curve Constructed with the integrated mortality frequency as ordinates  $L D_{50}$  has been given the value 0 in the graph Below, effect of lower doses, above, that of higher doses

### DISCUSSION

A critical study of the Knaff-Lenz method for the assay of digitalis based on 30 experiments on guinea-pigs has been made in the present paper. A frequency distribution chart and a dose-mortality curve have been worked out from these results. They closely resemble those of Trevan (*loc cit*) for strophanthin in frogs. The mean lethal dose of 9.07 c.c./kg body-weight of animal representing 1.129 units of digitalis activity compares favourably with the figure 17.98 c.c./kg representing 1.123 units, obtained in the same Laboratory in a previous series of observations on 20 cats but the standard deviation figure in guinea-pigs is definitely superior to that in cats ( $\pm 0.381$  in place of  $\pm 1.86$ ).

and the accuracy of the mean also is greater. These results do not appear to corroborate the findings of Gage (1933) who working with the old digitalis standard of 1926 on 11 guinea-pigs found a mean lethal dose of 12.40 c.c./kg body-weight of animal. The reason for this difference is not clear. Gage, however, used urethane subcutaneously and employed artificial respiration in his experiments. Whether these have significantly altered his m.l.d. figures or not is not possible to state.

*Advantages of the guinea-pig method of assay*—Special advantages of the guinea-pig method are in the simplicity and rapidity of the technique. The dissection of the jugular vein and the introduction of the venous cannula are extremely easy procedures. The most important thing for the experimenter is to keep a strict watch over the rate of flow of tincture of digitalis which can easily be done with the help of a capillary tube introduced into the perfusion burette. According to our experience, any irregularity in the flow of the perfusate is usually attended with variable results. The determination of the end-point by opening the thoracic cavity as usually recommended, is not necessary. The use of a needle for the same purpose is much simpler and avoids the possibility, while opening the thorax, of giving an artificial stimulation to the heart muscle, which may vitiate the accurate determination of the end-point.

The method is also very quick, and a complete experiment including both dissection and perfusion does not usually take more than 20 minutes. This is a real advantage, as by injecting urethane intraperitoneally to different animals at intervals of 20 minutes, one can easily perform from 8 to 10 experiments a day. This is not the case with the 'cat method' where hardly more than 2 experiments are practicable in a day. Hence experiments have to be spread over a number of days before a report can be given on a sample. Moreover, unlike cats, guinea-pigs are ideal laboratory animals which can easily be bred under controlled conditions.

One criticism that can, however, be made against the method is the failure in recording the systolic pressure of the animal during perfusion and consequent inability to study the cardiovascular effect of digitalis along with its toxicity. This can be done, if necessary, by devising a narrow-bore manometer but is considered superfluous by the authors as the palpatory method gives all the necessary information required for an accurate assay of digitalis.

### CONCLUSIONS

1. A simple method for the assay of digitalis in guinea-pigs has been described. The m.l.d. of International standard digitalis tincture in a series of 30 guinea-pigs is found to be  $9.07 \text{ c.c.} \pm 0.381$ . From the dose-mortality curve and the frequency distribution chart it appears that the method gives very dependable results.

2. In view of the easy availability, cheapness and the possibility of breeding guinea-pigs in all biological laboratories, the method should be suitable for routine laboratory examination of samples of tincture digitalis.

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## PARTITION OF PHOSPHORUS IN MAMMALIAN BRAINS

BY

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[Received for publication, June 24, 1942]

THE importance of phosphorus compounds in living tissues is well realized for the fact that they are concerned with the functioning of the central nervous system, with carbohydrate metabolism, with lipins and lipin metabolism, with the synthesis and breakdown of nucleic acids, and lastly suffer great changes in a large variety of diseased conditions. The phosphorus distribution in animal tissues is gaining great importance because the relative proportions of the various forms of phosphorus go a long way to suggest whether glycolysis in tissues takes place through a phosphorylating or a non-phosphorylating chain. There has been considerable agreement over the existence in certain tissues, for example, brain, tumour, embryo, etc., of a non-phosphorylating type of glycolysis, though the preparation of cell-free phosphorylating extracts from these tissues supports the view that an analogous phosphorylation takes place in the cell (Griger, 1940, Kerby and Bourne, 1940, Boyland and Bryland, 1935). The fact that glucose can act as a phosphate acceptor in dialysed brain dispersions (Ochoa, 1940) is even more convincing evidence of the correctness of this theory. MacFarlane and Weilherbe (1941), however, contrary to the conclusions of Needham *et al* (1937) and others, observe that glycolysis is dependent on phosphorylation.



The dry matter of the brain tissues of some of the vertebrates (dog, rat, rabbit, he-goat and ox) contains about 1.3 per cent of phosphorus. The present work was initiated to determine the distribution of various forms of phosphorus in brain tissues.

The important effect of the method of killing a tissue upon the resulting analytical figures was not realized until the publication of the work of Fletcher and Hopking (1907) on the lactic-acid content of the amphibian muscles. They showed the paramount importance of the rapid grinding and revealed the advantage of using ice-cold killing agents. It is now known that the true value for the inorganic ortho-phosphate cannot be obtained unless the tissue is cooled thoroughly before the grinding and ground rapidly in ice-cold reagents. Liquid air is sometimes used for the quick killing of small tissues but this technique though convenient is somewhat risky if the tissue is small (less than 200 mg).

The extraction and separation of the various forms of phosphorus by trichloro-acetic acid under preferably ice-cold conditions have been successively applied to a variety of tissues by Irving and Wells (1928) and we have adopted the procedure of Needham *et al* (*loc cit*) in this investigation.

#### EXPERIMENTAL.

*A Preparation of the material*—The brain tissues of ox, he-goat, dog, rabbit and rat were used for the fractionation of phosphorus. The whole brains of the ox and he-goat were obtained under ice-cold conditions from the slaughter-house immediately after the animals were killed. The brains of the dog and rabbit were taken after killing the animals by air injection in veins. The rats were killed by a blow on the head. The tissues were then trimmed free from the outer membranes and capillaries containing blood.

*B Methods*—The tissues were then weighed and a portion from each sample was kept in a tared evaporating basin in an air-oven at 103°C to 105°C, separately for moisture determination. The rest of the tissue was crushed in an ice-cold glass-mortar with a little silver-sand. They were then extracted with ice-cold 8 per cent trichloro-acetic acid for 30 minutes in the ice-bath with frequent stirring to facilitate extraction. The extractions were repeated thrice over.

The separation of the acid-soluble phosphorus into various fractions, namely, inorganic ortho-phosphate, labile esters such as adenylyl-pyrophosphate, resistant esters such as hexose-phosphates and triose-phosphates and phosphogens such as creatine-phosphate was carried out by the barium-precipitation method of Needham *et al* (*loc cit*).

The total phosphorus of each brain was determined from aliquots of the moisture-free material and calculated on the wet tissue. All phosphorus determinations were carried out colorimetrically according to the method of Fiske and Subbarow (1925).

The results of these experiments are presented in Tables I and II.—

TABLE I  
Phosphorus distribution in mammalian brains

Results are expressed in mg per 100 g of the wet tissue

Nature of P	Ox		Govt		Dog		Rabbit		Rat	
	Mg of P	Percent age of total P	Mg of P	Percent age of total P	Mg of P	Percent age of total P	Mg of P	Percent age of total P	Mg of P	Percent age of total P
Total P	349.90		105.00		312.00		300.10		331.00	
Barium precipitable										
Total acid soluble P	62.75	17.93	59.00	19.34	44.60	14.29	43.24	12.00	62.85	18.57
Total P	47.08	13.45	47.80	15.73	33.30	10.67	29.00	8.06	49.81	15.00
Inorganic P	31.90	9.12	43.80	14.30	20.80	9.55	23.09	6.41	41.28	13.00
Adenyl pyrophosphate P	2.88	0.85			1.10	0.45	3.15	0.90		
Hexo- and di-phosphate values	1.62	0.46	1.10	0.36	0.40	0.13	0.91	0.24		
Unhydrolysed after 100 minutes	10.05	3.05	2.90	0.95	1.70	0.55	1.85	0.51	6.73	1.97
Barium non precipitable										
Total P	15.54	4.44	11.25	3.69	11.01	3.53	13.76	3.82	12.50	3.75
Phosphogen P	4.21	1.20	3.10	1.01	1.80	0.58	3.41	0.95	3.21	0.90
Hexo mono phosphate values	11.33	3.24	8.11	2.68	9.21	2.95	10.35	2.87	8.29	2.75

TABLE II

*Phosphorus partition in brains*

Results expressed as percentage of total acid-soluble phosphorus

Nature of P	Ox	Goat	Dog	Rabbit	Rat
Barium precipitable					
Total P	75.03	81.02	74.10	67.10	79.20
Inorganic P	50.83	74.23	66.80	53.40	68.80
Adenyl-pyrophosphate P	4.59		3.14	7.30	
Hexose di-phosphate values	2.58	1.86	0.89	2.10	
Unhydrolysed P after 100 minutes	17.02	4.99	3.81	4.30	10.40
Barium non precipitable					
Total P	24.77	19.06	24.70	31.80	20.00
Phosphogen P	6.71	5.27	4.03	8.00	5.10
Hexose mono phosphate values	18.06	13.75	20.60	24.00	13.20

## CONCLUSIONS

The results indicate that all the five brains examined contain approximately the same proportions of different fractions of phosphorus

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## OBSERVATIONS ON THE BEHAVIOUR OF PULMONARY VESSELS IN THE ISOLATED PERFUSED RAT LUNGS

BY

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THE vasomotor responses in the isolated perfused guinea-pig lungs have been previously reported (Sinha, 1942) The present observations were made on the behaviour of the pulmonary vessels in the isolated perfused rat lungs Tribe (1912) working on the rat obtained vasoconstriction or vasodilatation respectively with adrenaline crystalline or adrenaline with chloretone HiraKawa (1925) noted no effect with adrenaline in the rat Foggie (1936) working on the rat with adrenaline crystalline found vasodilatation with small doses and vasoconstriction with large doses Acetylcholine caused vasoconstriction In the present work observations were made on the response of the pulmonary vessels with drugs and with nerve stimulation

### METHODS

The animal was killed by a blow on the neck, tracheotomy was done and a tracheal cannula was inserted, the chest opened and artificial respiration started Cannulae were then tied to the pulmonary artery and to the left auricle and perfusion was started immediately at a constant temperature and pressure, the perfusion pressure varying from 4 cm to 8 cm in different experiments The temperature was maintained at a constant level by passing the perfusion fluid from the reservoir through a glass-spiral, immersed in a bath, the temperature of which was kept at 40°C by a thermostatic relay arrangement, and thence to the inflow cannula The rate of outflow was recorded by a drop-recorder In the experiments on nerve excitation the stimulating electrode was placed in position before inserting the perfusion cannulae, as the nerves rapidly lose excitability once the perfusion has begun In all these experiments hypertonic tyrode solution was used (NaCl 0.8 g, KCl 0.02 g, CaCl<sub>2</sub> 0.01 g, MgCl<sub>2</sub> 0.01 g, NaH<sub>2</sub>PO<sub>4</sub> 0.005 g,

NaHCO<sub>3</sub> 0.1 g / 80 c.c distilled water) Drugs were injected by a syringe in the rubber-tubing connected with the inflow cannula

### EFFECT OF NERVE STIMULATION

1 *The thoracic sympathetic*—Electrical stimulation of the stellate ganglion was done in nineteen experiments. In fifteen experiments there was a decrease in the rate of flow showing vasoconstrictor effect (Fig 1a), in one there was an

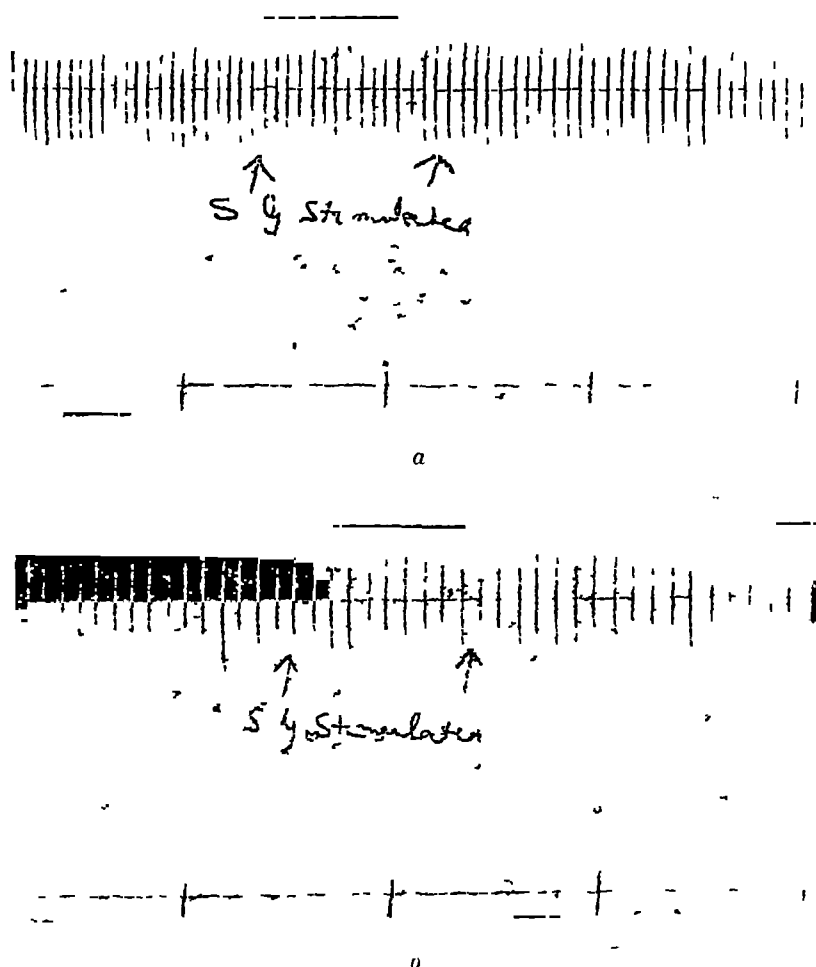


Fig 1 —16 10 41 —Effect of stimulation of the stellate ganglion on rate of outflow from perfused rat lungs. Stellate ganglion stimulated between arrows (a) before ergotamine, (b) 6 minutes after injection of 50 $\gamma$  ergotamine. In this and in Figs 2 and 3, the upper tracing shows the drop record of outflow, the lower tracing the time in minutes

increase in the rate of flow and in three there was no response. Previous injection of 50 $\gamma$  ergotamine suppressed or abolished the vasoconstrictor effect of the stellate-ganglion stimulation (Fig 1b).

The vasoconstriction observed by stimulation of the stellate ganglion goes to show the presence of vasoconstrictor nerve fibres in the thoracic sympathetic. The vasodilatation or absence of response observed in some of the experiments may be due either to the presence of vasodilator nerves or to the concomitant bronchodilatation leading to the stretching of the blood vessels in it. If there are vasodilator fibres in the thoracic sympathetic their control must be very feeble and it is also likely that they cease to function quickly under experimental conditions. The possibility of apparent vasodilatation as a result of concomitant bronchodilatation obtained support also by the observations of Dale and Narayana (1935).

2 *The vagi*—Electrical stimulation of the vagi was done in five experiments. In four of these experiments no effect was observed. Only in one experiment a decrease in the rate of flow was observed. It is likely that in the rat the vasomotor control through the vagus is slight, and if any such control at all exists it is lost under experimental conditions.

#### INJECTION OF DRUGS

*Adrenaline*—Adrenaline was injected in eleven experiments. In nine there was a decrease in the number of drops (Fig 2a), and in two there was an initial

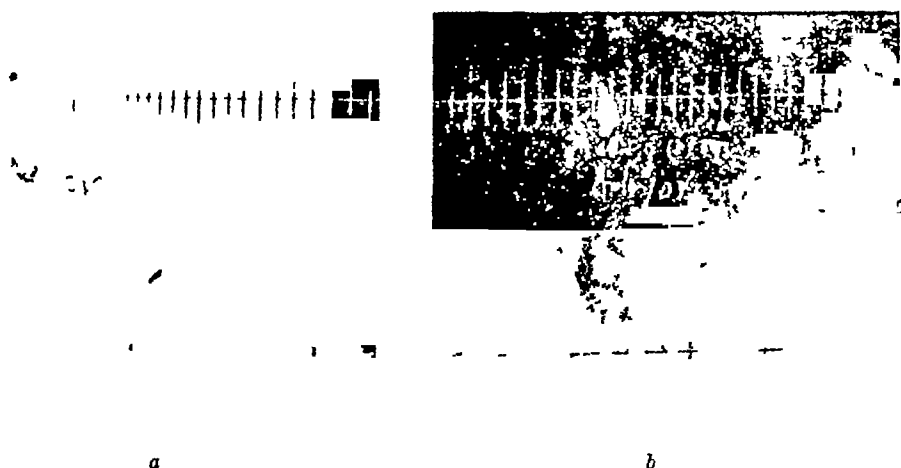


Fig 2—10 11 41—Effect of adrenaline on outflow from perfused rat's lungs and the abolition of the effect of adrenaline by previous injection of ergotamine. 10 $\gamma$  adrenaline injected at arrows (a) before ergotamine, (b) 7 minutes after injection of 50 $\gamma$  ergotamine

increase which on subsequent injection of adrenaline, showed a decrease in the number of drops. Injection of ergotamine suppressed or abolished the effect of adrenaline (Fig 2b)

In none of these experiments was vasoconstrictor response to adrenaline reversed by ergotamine. This is in contradiction to the observations of Foggie (*loc cit*)

*Acetylcholine*—In four experiments acetylcholine was injected and in each case there was a decrease in the number of drops (Fig 3a). This effect was abolished by previous injection of atropine (Fig 3b). The results obtained with acetylcholine confirm those of Foggie (*loc cit*)

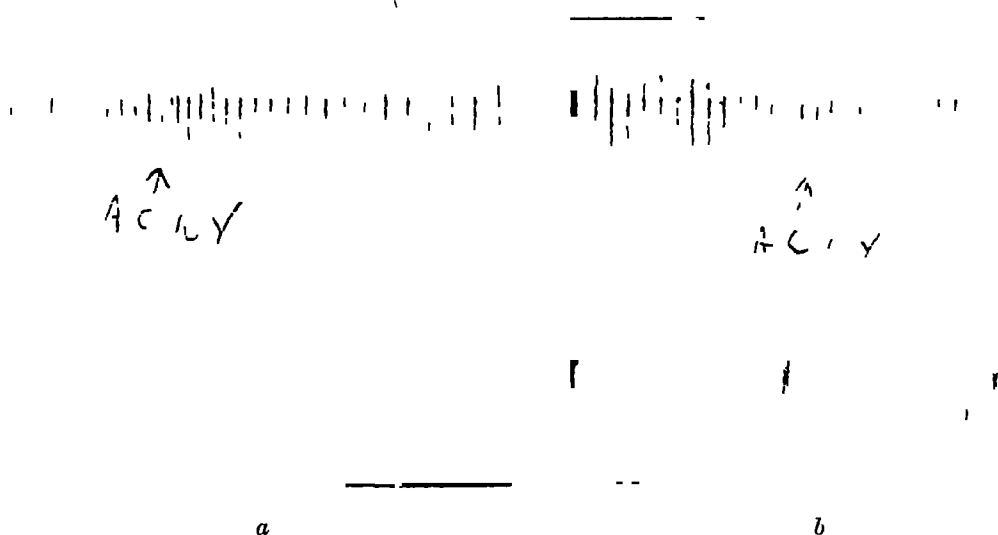


Fig 3—21 8 41—Effect of acetylcholine on outflow from perfused rat's lungs and the abolition of the effect of acetylcholine by previous injection of atropine. 10 $\gamma$  acetylcholine injected at arrows (a) before atropine, (b) 8 minutes after injection of 650 $\gamma$  atropine

### CONCLUSION

It is concluded from the above observations that the pulmonary blood vessels in the rat are supplied by vasoconstrictor fibres from the stellate ganglion as well as from the vagi, the control by the latter being feeble. The existence of vasodilator fibres in the thoracic sympathetic could not be established.

### SUMMARY.

- 1 Electrical stimulation of the stellate ganglion or injection of adrenaline caused vasoconstriction and the effect was abolished by previous injection of ergotamine.
- 2 Electrical stimulation of the vagi had very little vasoconstrictor effect.
- 3 Injection of acetylcholine also produced vasoconstriction which effect was abolished by previous injection of atropine.

## ACKNOWLEDGMENT

I wish to express my thanks to Professor B Narayana for his kind advice and encouragement throughout and for placing his laboratory facilities at my disposal

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## THE INTERACTION BETWEEN IONS DRUGS AND ELECTRICAL STIMULATION AS INDICATED BY INHIBITION IN UNSTRIATED MUSCLE

BY

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[Received for publication, August 10, 1942]

In previous papers (Singh, 1938, 1938a, 1938b, 1938c, 1938d, 1938e, 1939, 1939a, 1940, 1942) the properties of various contractions of unstriated muscle were studied, in the present research, the properties of inhibition were similarly investigated and also the inter-relation between the processes of excitation and inhibition.

Unstriated muscle is usually found in state of tonus which may be increased or diminished by various agencies. Tone is inhibited when the muscle is required to be relaxed, and when it has to perform quick contraction. It appears that tonic contraction is accompanied by increase in viscosity, and for quick performance such as a twitch, tone is inhibited and the viscosity reduced (Winton, 1937)

### EXPERIMENTAL

The experimental procedure was the same as described in previous papers, the pH of the saline was 6.5. The muscles used in these experiments were guinea-pig uterus, dog stomach and frog stomach, for study of inhibition the guinea-pig uterus was found most suitable. The majority of the experiments were thus performed on the guinea-pig uterus, and so a brief account of its reactions is given.

The reactions of the guinea-pig uterus are variable like those of *Mytilus* muscle. The guinea-pig uterus exhibits much tone greater than frog stomach, dog *Retractor penis* and *Mytilus* muscle. The pregnant uterus is more sensitive to chemical stimulation and less to electrical stimulation than the non-pregnant

one The excitability to alternating current (A. C.) or direct current (D. C.) is low, so that it was not possible to study the properties of the A. C. contraction in detail. The best method of stimulation with electric current is to increase the osmotic pressure of the medium to 1.4 to 1.8 times normal by adding sucrose in sodium chloride, thereby increasing the concentration of ions within the fibres. Adaptation to A. C. is slow just as in other mammalian plain muscle. The guinea-pig uterus is more sensitive to chemical stimulation than *Mytilus* muscle, frog stomach or dog stomach. Since the excitability to A. C. is low and adaptation is slow, this increased excitability of the guinea-pig uterus to chemical stimulation is due to two factors, viz. slow adaptation and the factor which affects the two excitabilities in opposite directions. The guinea-pig uterus produces a twitch, resembling the A. C. twitch when the muscle is transferred from saline to air and from air to saline. This tendency is also found to some extent in dog stomach and frog stomach, especially when the irritability to A. C. is high, as in the case of frog muscle immersed in saline rendered hypertonic with sucrose (osmotic pressure 1.3 to 1.4 times normal).

### INHIBITION

The guinea-pig uterus is markedly susceptible to inhibition and a considerable number of substances are active. Many agencies that produce contraction in other muscles produce inhibition in this muscle, so that the two phenomena are in some way related. Mere change of the same solution may cause inhibition, this renders it somewhat difficult to study the action of other substances, but means, which will be described later, were found to obviate this difficulty.

Some of the substances that produce inhibition in the guinea-pig uterus are (1) adrenaline from concentrations of 1 in 50 million, large concentrations may cause contraction, (2) anions small concentrations of iodide (0.0015 M NaI) is the only anion (out of Br, I, NO<sub>3</sub>, SCN) that produces marked inhibition, (3) cations all cations, such as hydrogen, lithium, sodium, potassium, ammonium, calcium, strontium, magnesium and barium, produce marked inhibition. Equivalent large concentrations of anions produce contraction, so that inhibition is a cation effect. The chemical substance chiefly used to study inhibition was potassium.

#### *Properties of the potassium inhibition*

Small concentrations of potassium are known to produce inhibition of plain muscle and correspondingly the latter may contract in a potassium-free saline, though the opposite also happens. Thus, *Mytilus* muscle contracts on withdrawal of potassium, while frog stomach relaxes, both these results are obtained with the guinea-pig uterus under varying conditions.

At pH 6.5 the muscle may slightly relax on withdrawal of potassium, but if after 10 minutes potassium is re-introduced it causes marked inhibition from which the muscle recovers. The muscle thus adapts to the inhibition, if it is deprived of potassium, this adaptation disappears. Just as contact with a stimulating

substance reduces the sensitivity of the muscle to the stimulus, contact with an inhibitory substance reduces the sensitivity to inhibition. In *Mytilus* muscle too it was found that after adaptation the effect of a stimulatory substance was reversed.

Just as a stimulatory substance exhibits the staircase effect a corresponding action is produced by an inhibitory substance, the successive magnitudes of inhibition increase. Fatigue of inhibition may also occur, especially under circumstances which produce adaptation to a stimulating agency. Thus, if tone gradually decreases owing to adaptation, fatigue to inhibition occurs.

The concentration of potassium in the mammalian saline is 0.00616 M KCl. After the muscle has been in a potassium-free saline for 10 minutes this concentration would produce the maximum inhibition, about 20 g to 30 g. The curve showing the relation between the concentration of potassium and the magnitude of inhibition is S-shaped.

In studying the effect of concentration on the potassium inhibition only sub-maximal concentrations should be used, the range used in these experiments was 0.00019 M KCl to 0.0016 M KCl. Large concentrations of potassium cause the guinea-pig uterus to contract, with subsequent stimulations, fatigue occurs and all concentrations of potassium cause inhibition only. This is important as it shows that potassium can produce similar effects within and without the fibres. In *Mytilus* muscle also, large concentrations of potassium cause complete inhibition.

In the study of the effect of substances on inhibition there are two distinct phenomena to be investigated, viz. the primary inhibition and the subsequent adaptation to the inhibition. The same agency may affect these two phenomena in the same or opposite directions. As with contraction, a decrease in inhibition may be due to rapidity of adaptation, so that to judge the effect of a substance on inhibition only the adaptation should also increase if the primary inhibitory increases.

*Effect of sodium chloride*—A change in the concentration of sodium chloride affects the potassium inhibition in a most significant way. If the sodium-chloride content of the saline is diminished (0 to 80 per cent of normal), the muscle relaxes instead of contracting on withdrawal of potassium. This is in agreement with the fact that the sodium-chloride content of *Mytilus* saline is high and *Mytilus* muscle contracts on withdrawal of potassium, and that the sodium-chloride content of frog saline is low and frog stomach relaxes on withdrawal of potassium.

Potassium antagonizes the action of sodium chloride, so that if the action of the latter is stimulatory, as in *Mytilus* muscle or sometimes in guinea-pig uterus (in alkaline solution pH 8, borate buffer), the muscle contracts on withdrawal of potassium, if the action of sodium chloride is inhibitory, such as in guinea-pig uterus immersed in saline the sodium-chloride content of which has been reduced, or in frog stomach, withdrawal of potassium causes relaxation.

In the guinea-pig uterus small concentrations of potassium produce a regular response in low concentrations of sodium chloride (15 per cent of normal), this method was used to study the potassium contraction

*Effect of cations*—Hydrogen and calcium ions increase the potassium inhibition as well as the subsequent adaptation. Lithium decreases the inhibition. This is probably due to increased adaptation, as lithium itself causes inhibition, inhibitory substances potentiate one another.

*Effect of anions and drugs*—Small concentrations (at pH 6.5) of bromide (0.007 M NaBr), iodide (0.0015 M NaI), thiocyanate (0.0004 M NaSCN) augment the potassium inhibition. Eserine ( $1$  in  $10^{-6}$ ) antagonizes potassium inhibition, smaller concentrations have no significant effect. Adrenaline potentiates the potassium inhibition, but if the muscle has adapted to adrenaline, potassium inhibition is also antagonized, adaptation to inhibition produced by one substance, is also antagonistic to that produced by another.

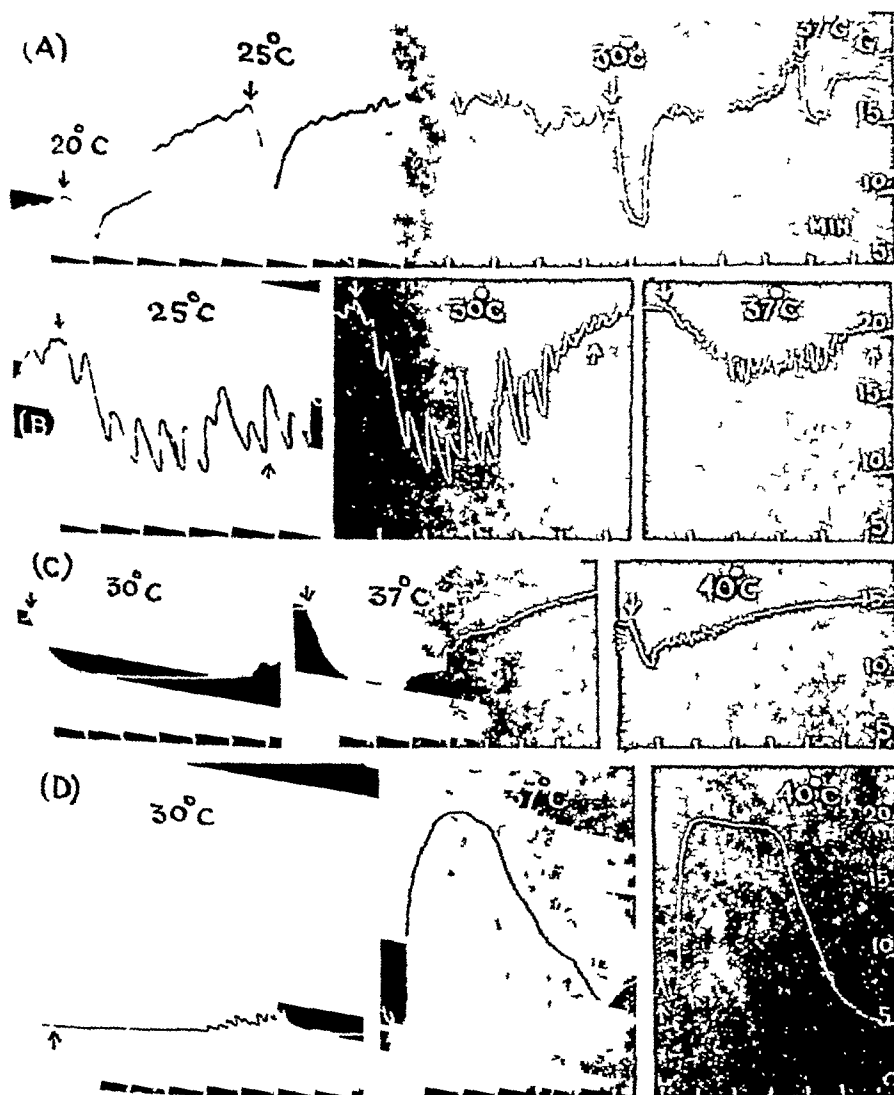
*Relation between tone and potassium inhibition*—Tone is antagonistic to potassium inhibition, the anions that cause tonic contraction are thus also antagonistic. Tonic contraction increases adaptation to the inhibition, calcium and hydrogen ions increase tone as well as hasten adaptation. Since in an adapted muscle, withdrawal of potassium causes relaxation, the latter increases tone. Hence though the initial inhibition increases with increase in the concentration of potassium (from 0.00019 M KCl to 0.0016 M KCl), adaptation also increases.

*The effect of temperature*—The optimum temperature for the potassium inhibition as well as the potassium contraction is  $35^{\circ}\text{C}$  to  $37^{\circ}\text{C}$ . The guinea-pig uterus, like *Mytilus* muscle, exhibits increased excitability to potassium at low temperature ( $5^{\circ}\text{C}$  to  $10^{\circ}\text{C}$ ). The decrease in inhibition as well as contraction at high temperature is due to increase in adaptation (Graph 1).

\* *Optimum length*—The optimum length for the potassium inhibition is the same as that for the potassium contraction.

*Effect of osmotic pressure*—About 10 to 20 per cent increase in osmotic pressure causes a marked inhibition so that it is difficult to judge the effect of such an increase on potassium inhibition as the base line does not remain constant, but further increase in osmotic pressure shows an antagonistic effect. If increase in osmotic pressure does not cause a marked inhibition, then small increase in osmotic pressure is also antagonistic (Graph 2). Since increase in osmotic pressure decreases tone, adaptation is also diminished. Judging from an electrical inhibition having the same properties as the potassium inhibition (to be described), small increase in osmotic pressure increases the inhibition in muscles which relax, this is an indirect effect due to neutralizing the antagonistic action of tone. Ions inside thus antagonize the action of ions outside. The above experiments show that certain agencies affect the actions of ions without the fibres similarly be it inhibitory or stimulatory. The implications of this statement has to be borne in mind when the effect of a solution

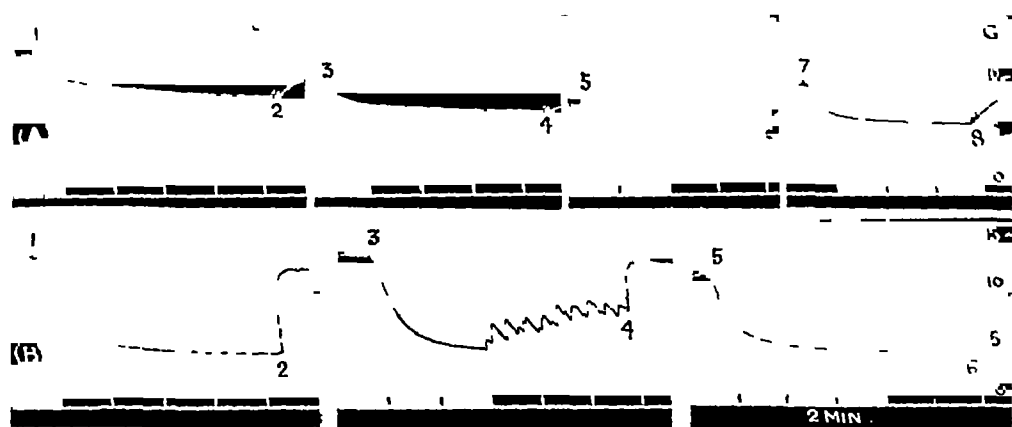
GRAPH 1



GRAPH 1—Guinea pig uterus. The effect of temperature on inhibition. (A) Inhibition by D.C. 8 V/1 mm. (B) Inhibition by increase in osmotic pressure to 1.05 times normal by adding sucrose. (C) Adaptation increases with temperature. (D) Inhibition by 0.00154 M KCl, adaptation increases with temperature. (E) Potassium contraction by 0.00610 M KCl in sodium chloride content 15 per cent of normal.

containing both inhibitory and stimulatory ions is to be considered. When the effects of both these increase or decrease simultaneously the net result will depend on their interaction.

GRAPH 2.



GRAPH 2—Guinea pig uterus. The effect of osmotic pressure on potassium inhibition. (A) Potassium (0.00616 M KCl) was added at 1, 3, 5, 7 and withdrawn at 2, 4, 6, 8. The 1st and the 4th figures show the effect of normal osmotic pressure, the 2nd that of osmotic pressure 1.2 times normal, and the 3rd that of 1.4 times normal. (B) 1st and 3rd figures show the effect of osmotic pressure (1.2 times normal), the 2nd figure in normal osmotic pressure, note rapid adaptation.

### *Mechanical inhibition*

Mere change of solution sometimes causes the muscle to relax. This inhibition resembles the potassium inhibition. It is not necessary for the muscle to pass from saline to air, a flow of saline through the chamber is sufficient to produce the inhibition. This is akin to the tonic mechanical contraction produced by touch in *Mytilus* muscle.

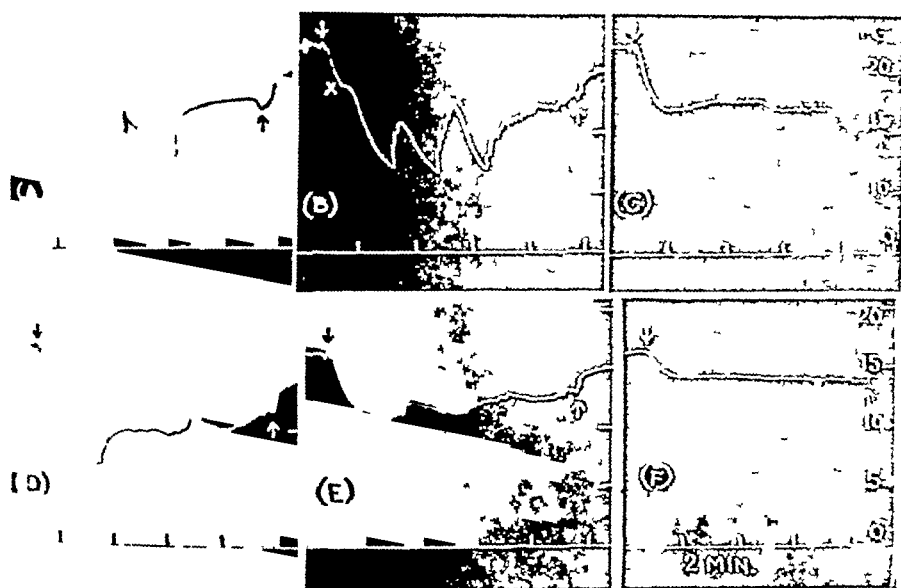
To obviate mechanical inhibition which sometimes interferes with these experiments, alkaline saline was employed (pH 8, borate buffer) or the chloride was replaced with bromide, as these are antagonistic to potassium inhibition.

### *Properties of the A C or D C inhibition.*

Electric current produces inhibition (a) during passage (b) on cessation, D C is more powerful than A C. The properties of this inhibition are as follows: (1) The optimum temperature is 27°C to 30°C, (2) the optimum length is less than that for potassium inhibition, (3) electrical inhibition is antagonistic to the potassium inhibition, A C however produces both kinds of inhibition (Graph 3), (4) hence cations, calcium, potassium, hydrogen, which cause inhibition, are antagonistic to the D C and one kind of A C inhibition, (5) tone is antagonistic both to electrical as well as potassium inhibition, but moderate increase in tone increases the A C or D C inhibition by antagonizing the action of cations in the saline, (6) anions are antagonistic to electrical inhibition, small concentrations because they

produce an antagonistic inhibition and larger concentrations because they cause tonic contraction, moderate concentrations causing slight increase in tone enhance the inhibition, (7) increase in osmotic pressure decreases the inhibition, as tone is decreased thereby

GRAPH 3



GRAPH 3—Guinea pig uterus. Antagonism between potassium and electrical inhibition. (A) Inhibition by 0.00154 M KCl (B) Inhibition by 0.00154 M KCl and A C 15 V/10 min. The antagonistic inhibition acts till  $\times$  where the synergistic inhibition begins (see Graphs 4 and 5) (C) Inhibition by 0.00154 M KCl and D C 8 V/10 min (D) Inhibition by 0.00154 M KCl only (E) Same as (C) (F) Inhibition by D C 8 V/10 min

*A C off-inhibition*—An interesting feature of the guinea-pig uterus is that the effect of electrical current resembles exactly that on *Mytilus* muscle except that inhibition is produced instead of contraction. Corresponding to the A C off-contraction there is the A C off-inhibition, which has the properties of the potassium inhibition, except that it is enhanced by increase in osmotic pressure. This difference is an indirect result of decrease of tone following increase in osmotic pressure. The optimum temperature is the same as that for tone (30°C to 35°C).

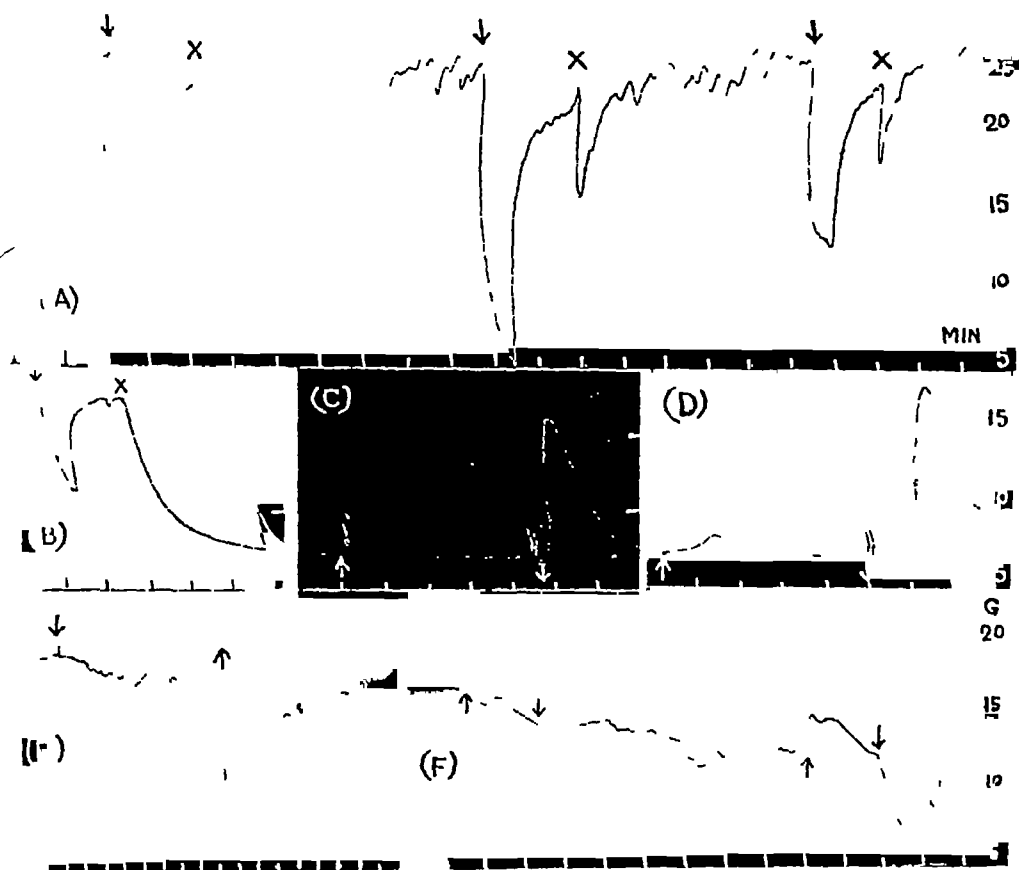
*The secondary A C or D C inhibition*—Corresponding to the secondary A C contraction there is the secondary A C or D C inhibition, having the properties of the potassium inhibition (Graph 4).

In *Mytilus* muscle A C (10 V/5 min) produces either (1) a twitch only or (2) if the sensitivity to ions outside is greater the twitch becomes smaller and is succeeded by a tonic contraction, or (3) if the sensitivity to ions outside is great then the



twitch disappears and only a tonic contraction results. The process which produced the A C contraction now merely exerts an antagonistic effect, which disappears on the cessation of the current, resulting in the second kind of A C off-contraction (Singh, 1940)

GRAPH 4

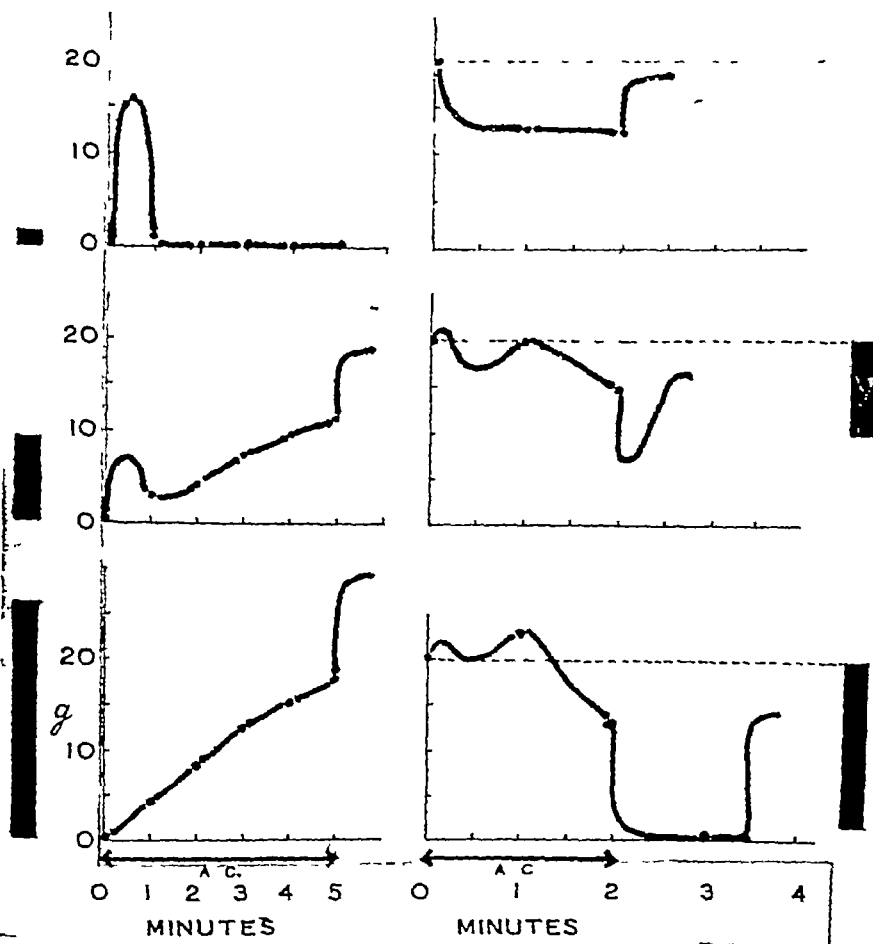


GRAPH 4—Inhibitions and contractions produced by electric current and increase in osmotic pressure (A) Guinea-pig uterus Primary D C inhibition at  $\downarrow$  and secondary D C inhibition at X, pH 8, borate buffer (B) Guinea pig uterus Same as (A) but pH 6, 5, the secondary D C inhibition is much more marked (C) and (D) Dog stomach Contraction produced by sudden increase in osmotic pressure at  $\uparrow$  and restoration to normal at  $\downarrow$  The contraction shown here on decreased osmotic pressure has a latent period of 1 min to 10 mins, so that it is akin to the secondary A C contracture (E) Guinea pig uterus Inhibition produced by increase in osmotic pressure at  $\uparrow$  to 1.1 times normal, and restoration of the original osmotic pressure at  $\downarrow$  (F) Guinea pig uterus The three inhibitions produced by A C (12 V/2 min) from  $\uparrow$  to  $\downarrow$  The first stimulation in saline containing potassium 0.00308 M KCl, the second in 0.00616 M KCl Note the A C off-inhibition

In the guinea-pig uterus, during the passage of A C increased sensitivity to ions outside results first in diminution of the A C inhibition and secondly in development of a secondary inhibition which has the properties of the potassium inhibition

On cessation of the current, there is the corresponding off-inhibition A C thus produces two kinds of inhibition (Graph 5)

GRAPH 5.



5a

5b

GRAPH 5a—*Mytilus* muscle (1) Contraction produced by A C 12V/5 min (2) in 1 in 100,000 NaCN (3) in 1 in 10,000 NaCN, the twitch has disappeared and only the tonic contraction is produced

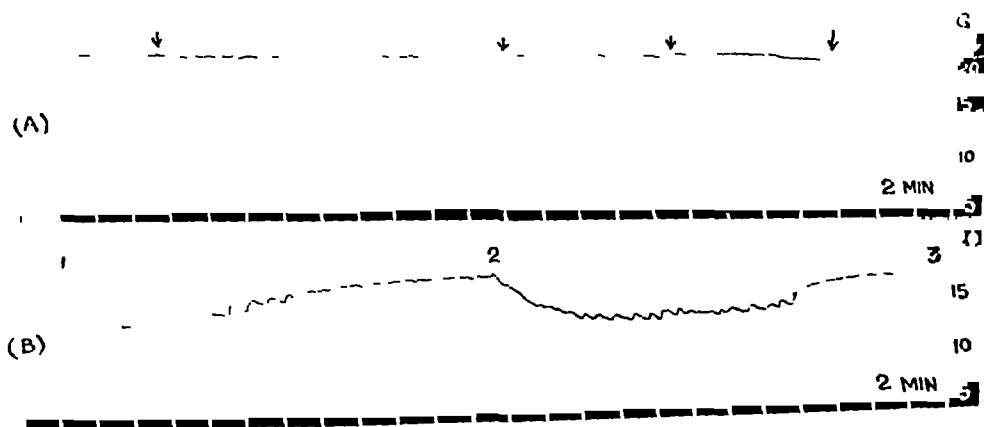
GRAPH 5b—Guinea-pig uterus (1) Inhibition by A C 12V/2 min. in potassium free saline (2) in 0.00308 M KCl (3) in 0.00616 M KCl The A C off inhibition increases with potassium Columns on the left-hand side show tension produced by 0.1 M KCl Columns on the right-hand side show inhibition produced by similar quantity of potassium The muscle was stimulated with A C. after it had recovered from the potassium inhibition Thick lines below show period of stimulation with A C

The question arises as to why should, in the guinea-pig uterus, A C or D C produce inhibition, this is due to the increased tone in this muscle. If A C or D C produces a twitch, they by substituting the chloride of the saline with bromide increase the tone, and electric current only produces inhibition, bromide neutralizes the effect of cations outside. In the guinea-pig uterus during the passage of A C the sequence of events may be as follows: first, a twitch, which is succeeded by inhibition, adaptation occurs to this, and then, the second inhibition. The sequence of events following the twitch is due to increased sensitivity to ions outside during the passage of the current. Increased sensitivity, probably to anions, results in the first inhibition, and to cations results in recovery and the succeeding inhibition. As with the A C contraction in *Mytilus* muscle the A C inhibition in the guinea-pig uterus has an optimum voltage. This is partly due to the fact that increase in voltage simultaneously increases the action of factors which are antagonistic to each other. Increased action of ions outside decreases the A C contraction in *Mytilus* muscle and the A C inhibition in guinea-pig uterus.

#### *Withdrawal inhibition.*

Withdrawal of certain stimulating substances produces inhibition. These are bromide, iodide, acetylcholine, potassium. This inhibition is probably due to adaptation, the muscle producing an opposite change (Graph 6).

GRAPH 6



GRAPH 6—Guinea pig uterus Withdrawal inhibition (A) At the first arrow was added acetylcholine (1 in 100,000), the same solution was renewed at each arrow, when no inhibition took place (B) At 1 the acetylcholine was replaced with saline, this caused inhibition. At 2, the saline was renewed, the inhibition is due to the fact that first replacement with saline does not remove the acetylcholine completely. At 3, renewal of the saline caused no change.

Withdrawal of sodium chloride by replacement with sucrose causes a temporary inhibition. This is due to the withdrawal of chloride, as other halides produce this

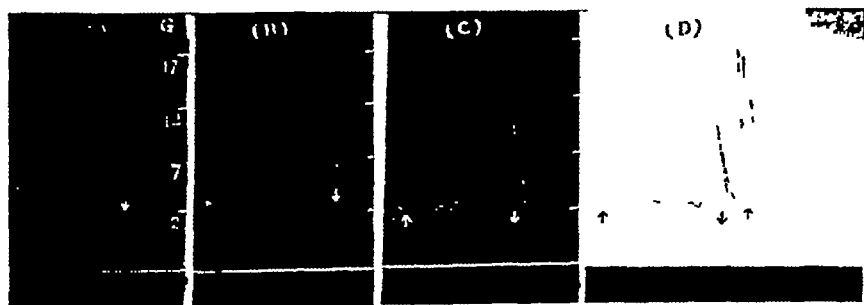
effect, besides, replacement of sodium with lithium does not result in such marked inhibition the concentration of chloride thus remaining constant

*Properties of the inhibition and the contraction produced by increase of osmotic pressure*

*Properties of the osmotic contraction*—Frog muscle is more sensitive to A C than dog stomach or guinea-pig uterus, the sensitivity of these muscles to sudden increase in osmotic pressure also varies in the same order, hence the osmotic contraction was studied in the frog muscle

Changes in osmotic pressure produce two distinct contractions sudden increase in osmotic pressure produces a contraction, if the osmotic pressure is suddenly restored to normal another contraction occurs. The contraction produced by increase in osmotic pressure has the properties of the A C contraction, the contraction produced on restoration of normal osmotic pressure is antagonistic and has the properties of the A C off-contraction, as shown by the following experiments (Graph 7) —

GRAPH 7



GRAPH 7—Frog stomach muscle. Contraction produced by sudden increase in osmotic pressure at  $\uparrow$  and restoration of original osmotic pressure at  $\downarrow$ . (A) First response (B) Staircase effect, the first osmotic contraction becomes larger and the second smaller (C) The effect of 0.02 M  $\text{NH}_4\text{Cl}$  (D) the effect of 0.01 M KCl and then 0.006 M NaI

(1) The optimum concentration of calcium for the first osmotic contraction is the same as that for the A C contraction, that for the second osmotic contraction is the same as that for potassium (2) The optimum length for potassium in frog *Mytilus* and dog muscles is greater than that for A C contraction. The optimum length for the first osmotic contraction is the same or slightly less than that for the A C contraction, so that the optimum length for the osmotic contraction is more equal to that for the A C contraction than that for potassium contraction (3) The optimum temperature for the osmotic as well as the A C contraction is  $30^\circ\text{C}$  (4) If the concentration of stimulating ions in the saline is increased by adding ammonium or potassium the first osmotic contraction is depressed and the second

increased, just as the A C off-contraction (3) In dog stomach calcium and hydrogen ions augment the second osmotic contraction just as they do the potassium contraction

Another interesting feature is that a contraction with a latent period of 1 to 10 minutes occurs when the osmotic pressure is restored to normal This is best seen in the dog stomach and is akin to the secondary A C contraction A similar contraction occurs if the concentration of calcium is increased and then restored to normal It has been presumed that the secondary A C contraction follows changes in the concentration of calcium in the muscle (Singh, 1938a)

Thus, the three contractions produced by passage of electric current can also be produced by sudden increase in osmotic pressure In frog muscle, the contraction produced by mere change of saline should not be confused with the osmotic contraction, the magnitude of the former is determined by merely changing the same saline

*Properties of the osmotic inhibition* — This was studied in the guinea-pig uterus Just as the passage of electric current produces two inhibitions, one during the passage and the other on cessation, so also do changes in osmotic pressure Inhibition is produced if the osmotic pressure is increased and also when the osmotic pressure is restored to normal

The properties of the inhibition produced on increase of osmotic pressure are as follows (1) as with A C or D C inhibition, the optimum temperature is 30°C, (2) it is antagonized by hydrogen and calcium ions, (3) unlike A C or D C inhibition it is not antagonized by potassium, this is probably due to increase in tone on withdrawal of potassium, (4) as with the A C inhibition, deficiency in sodium chloride increases the osmotic inhibition

In frog muscle sudden increase in osmotic pressure causes relaxation followed by contraction Small increase in osmotic pressure only causes relaxation With A C too, in *Mytilus* muscle, small voltage causes relaxation, large voltage causes contraction, so that there is an optimum voltage for inhibition This appears to be due to the fact that increase in concentration of the ions within the fibres first neutralizes tone, and then produces contraction, so that there is also an optimum value for increase in osmotic pressure to produce inhibition In guinea-pig uterus increase in osmotic pressure by 10 to 20 per cent above normal is sufficient to produce the maximum inhibition, further increase may cause contraction

### TONUS

The guinea-pig uterus exhibits much tone, and yet at times it may be completely relaxed *Mytilus* muscle exhibits much tone, while frog muscle exhibits very little The reasons for these variations will now be described —

*Effect of sodium chloride* — In previous papers (Singh, 1938 to 1938e, 1939, 1939a) it has been described that large concentrations of sodium chloride have a stimulatory while smaller concentrations have inhibitory effect, and that tone is due to the stimulating effect of sodium chloride, this is very well shown by

the guinea-pig uterus. Partial replacement of the sodium chloride of the saline with sucrose decreases tone. In saline containing sodium chloride in the same concentrations as in frog saline, tone is greatly diminished, in agreement with this is the fact that frog muscle exhibits much less tone than the guinea-pig uterus. If the sodium chloride is reduced below 20 per cent of normal concentrations then tone again increases, showing the inhibitory effect of small concentrations of sodium chloride. Tone in isolated muscle is thus produced by sodium chloride and uses up oxygen (Rao and Singh, 1940).

Isolated *Mytilus* muscle usually however exhibits tone quite different from that of isolated guinea-pig uterus, frog stomach or dog stomach, the spontaneous contractions are absent. The tone of *Mytilus* muscle is a slow relaxation due to increased viscosity, this latter depresses excitability and so the spontaneous contractions are absent. Pig stomach exhibits tone like that of *Mytilus* muscle and the latter sometimes exhibits tone like that of the guinea-pig uterus.

*Effect of lithium*—In frog stomach and dog stomach, lithium increases and in guinea-pig uterus decreases tone. This is due to the inhibitory effect of lithium in guinea-pig uterus. If the sodium-chloride content of the saline is reduced then the guinea-pig uterus behaves like frog muscle, that is, smaller concentrations of sodium have a greater inhibitory effect than lithium. This finding is important, because the concentration of chloride remains constant suggesting that sodium, like other cations, has an inhibitory effect.

*The effect of other cations, calcium, potassium and hydrogen ions*—This has been described previously. Sudden increase in hydrogen ions produces inhibition like that of potassium, the muscle adapts to a slow increase.

*Effect of temperature*—The guinea-pig uterus, like *Mytilus* muscle, shows increased tone at low temperature (below 10°C). It may show increased tone at 20°C to 25°C and at 35°C to 37°C as in *Mytilus* frog and dog muscles or it may be completely relaxed.

*Effect of stretching*—In a previous paper (Rao and Singh, *loc. cit.*) it has been shown that the oxygen consumption of frog stomach decreased if the length was increased, and this was ascribed to the antagonistic action of stretching on tone, this has been found to be the case.

To explain these complicated phenomena, such as the effect of stretching and the variable effect of temperature, it must be remembered that the saline contains two antagonistic factors, one that produces contraction and the other relaxation, both these factors have the same optimum length and optimum temperature so that when these two factors are varied, the net result depends upon the predominance of one factor over the other.

*Effect of ionic composition of the muscle*—In previous papers it has been mentioned that plain muscle exhibits greater tone than striated muscle owing to its greater sodium content. This conclusion finds support from the present experiments. The inhibitory effect of potassium is greater than that of sodium so that the muscle will show greater tone if it contains less potassium and more sodium.

increased, just as the A C off-contraction (3) In dog stomach calcium and hydrogen ions augment the second osmotic contraction just as they do the potassium contraction

Another interesting feature is that a contraction with a latent period of 1 to 10 minutes occurs when the osmotic pressure is restored to normal This is best seen in the dog stomach and is akin to the secondary A C contraction A similar contraction occurs if the concentration of calcium is increased and then restored to normal It has been presumed that the secondary A C contraction follows changes in the concentration of calcium in the muscle (Singh, 1938a)

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results (5) Interaction between ions within and without on the one hand and the factor of adaptation on the other, which is probably calcium

Usually two kinds of spontaneous contractions are seen (1) in relaxed muscle and (2) in contracted muscle. In relaxed muscle they are produced by alternate contraction and in contracted muscle, they are produced by alternate relaxation, the guinea-pig uterus usually exhibits these latter kind of spontaneous contractions (Graph 8)

### *Withdrawal contractions*

The guinea-pig uterus contracts on withdrawal of a number of substances, such as calcium, potassium (large and small concentrations), strontium, ammonium, and thiocyanate. The adrenaline withdrawal contraction in the dog stomach is enhanced by calcium and hydrogen ions, and so like other withdrawal contractions belongs to the potassium group, that is, produced by ions outside. This view is supported by the fact that adrenaline produces a withdrawal contraction only if the muscle is sensitive to calcium and hydrogen ions.

*Effect of ions on weight of mammalian muscle*—The effect of ions on the dog stomach resembles that on the frog stomach. Isotonic solution of sodium chloride causes the muscle to lose weight by about 5 to 10 per cent, potassium and calcium antagonize this. The guinea-pig uterus behaves like *Mytilus* muscle, isotonic solution of sodium chloride causes the muscle to gain weight.

These changes in weight vary in the same order as the physiological activities of these muscles. Both in *Mytilus* muscle and the guinea-pig uterus, the effect of sodium chloride is stimulatory and these muscles gain weight in sodium chloride. In dog stomach and frog stomach the effect is inhibitory and these muscles lose weight. In frog stomach, the inhibition is greater than dog stomach and the former loses more weight (20 per cent) than the latter (10 per cent).

### DISCUSSION

The effect of the sodium and the chloride of the mammalian saline on the guinea-pig uterus appears to be antagonistic, sodium, like other cations, has an inhibitory effect and chloride like other anions, a stimulatory effect. Calcium increases tone by antagonizing the effect of sodium, greater increase in the concentration of calcium decreases tone by neutralizing the effect of chloride. Withdrawal of sodium chloride sometimes causes relaxation due to withdrawal of chloride and sometimes contraction due to withdrawal of sodium. Sodium, like other cations, increases the excitability to electric current. A decrease in this excitability following withdrawal of sodium chloride is due to the withdrawal of sodium and increase to withdrawal of chloride.

The natural tendency for myosin molecules is to contract. To keep them extended, energy is required, this is in agreement with the fact that in one kind of lengthening of plain muscle, the oxygen consumption is increased (Rao and Singh *loc cit*). It is the function of inhibition, presumably by sodium chloride to keep

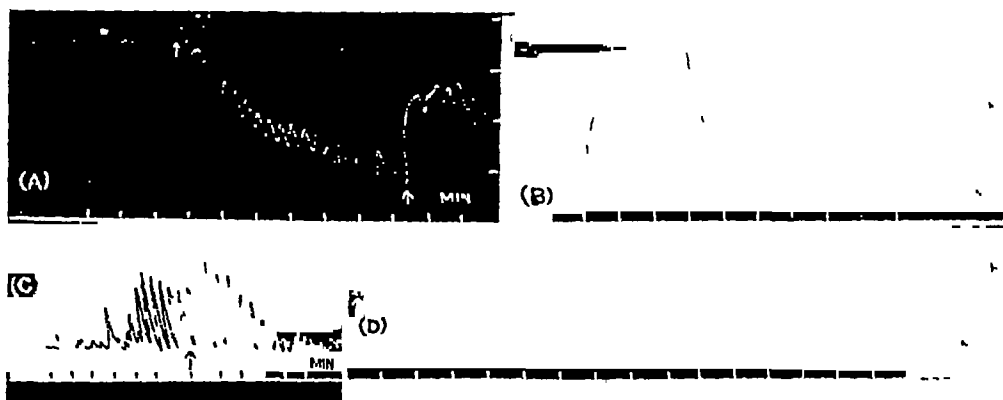


*Spontaneous contractions*

These are produced when two systems of nearly equal potency, one inhibitory and the other excitatory, interact. Thus, the inhibitory systems in plain muscle are (a) ions within the fibres, (b) cations in the saline and (c) calcium, the excitatory systems are also (a) ions within the fibres and (b) ions without the fibres.

The spontaneous contractions can thus be produced by the following methods  
 (1) Interaction between ions inside and outside. If the muscle is in tone, then increase in osmotic pressure, not sufficient to produce complete inhibition, produces spontaneous contractions. If the muscle is relaxed, then contracture can be induced by potassium and increase in osmotic pressure would produce spontaneous contractions. Instead of change of osmotic pressure, A.C. can be employed.  
 (2) Interaction between ions within and calcium. Increase in osmotic pressure may result in increased tone, moderate increase in calcium concentration will result in spontaneous contractions.  
 (3) Interaction between ions outside and calcium.

GRAPH 8



GRAPH 8—Guinea pig uterus. Various kinds of spontaneous contractions. (A) Inhibition produced by 0.00154 M KCl at  $\downarrow$ , potassium withdrawn at  $\downarrow$ . Note that one kind of spontaneous contractions is superimposed on the inhibition curve, as the muscle relaxes, they increase in magnitude, as happens with the response to A.C. These contractions are similar to those found in frog stomach. (B) The muscle is in tone, and it alternately relaxes, this spontaneous inhibition has the same properties as the potassium inhibition, this is the result of the interaction between anions and cations of the saline. (C) Spontaneous contractions produced by tetra methylammonium bromide the substance withdrawn at  $\downarrow$ . (D) Contraction produced by 0.00616 M KCl in sodium chloride 15 per cent of normal. Spontaneous contractions are produced as a result of interaction between potassium and the factor of adaptation.

Many substances that produce tonic contraction produce spontaneous contraction if the concentration of calcium is increased, the tone of the guinea-pig uterus is affected in the same way. (4) Interaction between anions and cations of the saline. This can be produced by properly adjusting their concentration, or by stretching, which increases the action of either. Increase in temperature produces similar

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it relaxed. Hence when the muscle shortens, this energy will be liberated as heat, and thus account for the heat of contraction or the Fern effect.

### SUMMARY

1 Inhibition is well marked in the guinea-pig uterus. Three agencies produce inhibition: (1) chemical substances, (2) electric current and, (3) increase in osmotic pressure, that is, ions within the muscle fibres. Just as the contraction produced by electric current is antagonistic to that produced by potassium, so also are the inhibitions produced by these agencies. Increase in osmotic pressure produces most of the phenomena produced by electric current. Alternating current produces two kinds of contraction as well as inhibition.

2 Plain muscle exhibits two kinds of tone, one due to slow relaxation and the other to the contraction produced by chloride. The action of sodium and chloride in the saline is antagonistic.

3 There are two kinds of spontaneous contractions: one due to alternate contractions and the other to alternate relaxation.

### ACKNOWLEDGMENT

I wish to thank Lieut-Colonel S. S. Sokhey, I.M.S., Director, Haffkine Institute, Bombay, for the facilities provided.

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LONDON H. K. LEWIS & CO., LTD, 136, GOWER STREET, W.C.1.

## ANNALS OF TROPICAL MEDICINE and PARASITOLOGY

Issued by the Liverpool School of Tropical Medicine

Edited by Professor WARRINGTON YORKE, MD, FRCP, FRS  
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Annual Subscription £1 2 6, post free

Vols I-XXXIII, 1907-1939 £1 2 6 per Vol., unbound (a few of the back numbers are out of print)

UNIVERSITY PRESS OF LIVERPOOL

177, Brownlow Hill, Liverpool 3

